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(54) **Consensus phytases**

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Description

[0001] Phytases (*myo*-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8) are enzymes that hydrolyze phytate (*myo*-inositol hexakisphosphate) to *myo*-inositol and inorganic phosphate and are known to be valuable feed additives.

[0002] A phytase was first described in rice bran in 1907 [Suzuki et al., Bull. Coll. Agr. Tokio Imp. Univ. 7, 495 (1907)] and phytases from Aspergillus species in 1911 [Dox and Golden, J. Biol. Chem. 10, 183-186 (1911)]. Phytases have also been found in wheat bran, plant seeds, animal intestines and in microorganisms [Howsen and Davis, Enzyme Microb. TechnoL 5, 377-382 (1983), Lambrechts et al., Biotech. Lett. 14, 61-66 (1992), Shieh and Ware, Appl. Microbiol. 16, 1348-1351 (1968)].

[0003] The cloning and expression of the phytase from Aspergillus niger (ficuum) has been described by Van Hartingsveldt et al., in Gene, 127, 87-94 (1993) and in European Patent Application, Publication No. (EP) 420 358 and from Aspergillus niger var. awamori by Piddington et al., in Gene 133, 55-62 (1993).

[0004] Cloning, expression and purification of phytases with improved properties have been disclosed in EP 684 313. However, since there is a still ongoing need for further improved phytases, especially with respect to their thermostability, it is an object of the present invention to provide a consensus phytase by the following process.

[0005] A process for the preparation of a consensus protein, whereby such process is characterized by the following steps:

a) at least three preferably four amino acid sequences of a defined protein family are aligned by any standard alignment program known in the art;

b) amino acids at the same position according to such alignment are compared regarding their evolutionary similarity by any standard program known in the art, whereas the degree of similarity provided by such a program which defines the least similarity of the amino acids that is used for the determination of an amino acid of corresponding positions is set to a less stringent number and the parameters are set in such a way that it is possible for the program to determine from only 2 identical amino acids at a corresponding position an amino acid for the consensus protein; however, if among the compared amino acid sequences are sequences that show a much higher degree of similarity to each other than to the residual sequences, these sequences are represented by their consensus sequence determined as defined in the same way as in the present process for the consensus sequence of the consensus protein or a vote weight of 1 divided by the number of such sequences is assigned to every of those sequences.

c) in case no common amino acid at a defined position can be identified by the program, any of the amino acids of all sequences used for the comparison, preferably the most frequent amino acid of all such sequences is selected or an amino acid is selected on the basis of the consideration given in Example 2.

d) once the consensus sequence has been defined, such sequence is back-translated into a DNA sequence, preferably using a codon frequency table of the organism in which expression should take place;

e) the DNA sequence is synthesized by methods known in the art and used either integrated into a suitable expression vector or by itself to transform an appropriate host cell;

f) the transformed host cell is grown under suitable culture conditions and the consensus protein is isolated from the host cell or its culture medium by methods known in the art.

process step b) can also be defined as follows:

b) amino acids at the same position according to such an alignment are compared regarding their evolutionary similarity by any standard program known inthe art, whereas the degree of similarity provided by such program is set at the lowest possible value and the amino acid which is the most similar for at least half of the sequences used for the comparison is selected for the corresponding position in the amino acid sequence of the consensus protein.

[0006] This whole process can be seen in a process in which a sequence is choosen from a number of highly homologous sequences and only those amino acid residues are replaced which clearly differ from a consensus sequence of this protein family calculated under moderately stringent conditions, while at all positions of the alignment where the method is not able to determine an amino acid under moderately stringent conditions the amino acids of the preferred

sequence are taken.

[0007] The program used for the comparison of amino acids at a defined position regarding their evolutionary similarity is the program "PRETTY". The defined protein family is the family of phytases, especially wherein the phytases are of fungal origin.

[0008] The host cell is of eukaryotic, especially fungal, preferably Aspergillus or yeast, preferably Saccharomyces or Hanseaula origin.

[0009] It is an object of the present invention to provide a consensus protein obtainable preferably obtained, by such processes and specifically the consensus protein, which has the amino acid sequence shown in Figure 2 or a variant thereof. A "variant" refers in the context of the present invention to a consensus protein with amino acid sequence shown in Figure 2 wherin at one or more positions amino acids have been deleted, added or replaced by one or more other amino acids with the provisor that the resulting sequence provides for a protein whose basic properties like enzymatic activity (type of and specific activity), thermostability, activity in a certain pH-range (pH-stability) have not significantly been changed. "Signaficantly means in this context that a man skilled in the art would say that the properties of the variant may still be different but would not be unobvious over the ones of consensus protein with the amino acid sequence of Figure 2 itself.

A mutein refers in the context of the present invention to replacements of the amino acid in the amino acid sequences of the consensus proteins shown in

Figure 2 which lead to consensus proteins with further improved properties e. g. activity. Such muteins can be defined and prepared on the basis of the teachings given in European Patent Application number 97810175.6, e. g. Q50L, Q50T, Q50G, Q50L-Y51N, or Q50T-Y51N. "Q50L" means in this context that at position 50 of the amino acid sequence the amino acid Q has been replaced by amino acid L.

[0010] In addition, a food, feed or pharmaceutical composition comprising a consensus protein as defined above is also an object of the present invention.

[0011] In this context "at least three preferably three amino acid sequences of such defined protein family" means that three, four, five, six to 12, 20, 50 or even more sequences can be used for the alignment and the comparison to create the amino acid sequence of the consensus protein. "Sequences of a defined protein family" means that such sequences fold into a three dimensional structure, wherein the α-helixes, the β-sheets and-turns are at the same position so that such structures are, as called by the man skilled in the art, superimposable. Furthermore these sequences characterize proteins which show the same type of biological activity, e.g. a defined enzyme class, e.g. the phytases. As known in the art, the three dimensional structure of one of such sequences is sufficient to allow the modelling of the structure of the other sequences of such a family. An example, how this can be effected, is given in the Reference Example of the present case. "Evolutionary similarity" in the context of the present invention refers to a schema which classifies amino acids regarding their structural similarity which allows that one amino acid can be replaced by another amino acid with a minimal influence on the overall structure, as this is done e.g. by programs, like "PRETTY", known in the art. The phrase "the degree of similarity provided by such a program...is set to less stringent number" means in the context of the present invention that values for the parameters which determine the degree of similarity in the prgram used in the practice of the present invention are chosen in a way to allow the program to define a common amino acid for a maximum of positions of the whole amino acid sequence, e. g. in case of the program PRETTY a value of 2 or 3 for the THRESHOLD and a value of 2 for the PLURALITY can be choosen.

Furthermore, "a vote weight of one devided by the number of such sequences" means in the context of the present invention that the sequences which define a group of sequences with a higher degree of similarity as the other sequences used for the determination of the consensus sequence only contribute to such determination with a factor which is equal to one devided by a number of all sequences of this group.

As mentioned before should the program not allow to select the most similar amino acid, the most frequent amino acid is selected, should the latter be impossible the man skilled in the art will select an amino acid from all the sequences used for the comparison which is known in the art for its property to improve the thermostability in proteins as discussed e.g. by.

Janecek, S. (1993), *Process Biochem.* 28, 435-445 or

Fersht, A. R. & Serrano, L. (1993). *Curr. Opin. Struct. Biol.* 3, 75-83.

Alber, T. (1989), *Annu. Rev. Biochem.* 58, 765-798 or

Matthews, B. W. (1987), *Biochemistry* 26, 6885-6888.

Matthews, B. W. (1991), *Curr. Opin. Struct. Biol.* 1, 17-21.

[0012] The stability of an enzyme is a critical factor for many industrial applications. Therefore, a lot of attempts, more or less successful, have been made to improve the stability, preferably the thermostability of enzymes by rational (van den Burg *et al.*, 1998) or irrational approaches (Akanuma *et al.*, 1998). The forces influencing the thermostability of a protein are the same as those that are responsible for the proper folding of a peptide strand (hydrophobic interactions, van der Waals interactions, H-bonds, salt bridges, conformational strain (Matthews, 1993). Furthermore, as shown by Matthews *et al.* (1987), the free energy of the unfolded state has also an influence on the stability of a protein.

Enhancing of protein stability means to increase the number and strength of favorable interactions and to decrease the number and strength of unfavorable interactions. It has been possible to introduce disulfide linkages (Sauer *et al.*, 1986) to replace glycine with alanine residues or to increase the proline content in order to reduce the free energy of the unfolded state (Margarit *et al.*, 1992; Matthews, 1987a). Other groups concentrated on the importance of additional H-bonds or salt bridges for the stability of a protein (Blaber *et al.*, 1993) or tried to fill cavities in the protein interior to increase the buried hydrophobic surface area and the van der Waals interactions (Karpusas *et al.*, 1989). Furthermore, the stabilization of secondary structure elements, especially α-helices, for example, by improved helix capping, was also investigated (Munoz & Serrano, 1995).

[0013] However, there is no fast and promising strategy to identify amino acid replacements which will increase the stability, preferably the thermal stability of a protein. Commonly, the 3D structure of a protein is required to find locations in the molecule where an amino acid replacement possibly will stabilize the protein's folded state. Alternative ways to circumvent this problem are either to search for a homologous protein in a thermo- or hyperthermophile organism or to detect stability-increasing amino acid replacements by a random mutagenesis approach. This latter possibility succeeds in only 10^3 to 10^4 mutations and is restricted to enzymes for which a fast screening procedure is available (Arase *et al.*, 1993; Risse *et al.*, 1992). For all these approaches, success was variable and unpredictable and, if successful, the thermostability enhancements nearly always were rather small.

[0014] Here we present an alternative way to improve the thermostability of a protein. Imanaka *et al.* (1986) were among the first to use the comparisons of homologous proteins to enhance the stability of a protein. They used a comparison of proteases from thermophilic with homologous ones of mesophilic organisms to enhance the stability of a mesophilic protease. Serrano *et al.* (1993) used the comparison of the amino acid sequences of two homologous mesophilic RNases to construct a more thermostable Rnase. They mutated individually all of the residues that differ between the two and combined the mutations that increase the stability in a multiple mutant. Pantoliano *et al.* (1989) and, in particular, Steipe *et al.* (1994) suggested that the most frequent amino acid at every position of an alignment of homologous proteins contribute to the largest amount to the stability of a protein. Steipe *et al.* (1994) proved this for a variable domain of an immunoglobulin, whereas Pantohano *et al.* (1989) looked for positions in the primary sequence of subtilisin in which the sequence of the enzyme chosen to be improved for higher stability was singularly divergent. Their approach resulted in the replacement M50F which increased the T_m of subtilisin by 1.8 °C.

[0015] Steipe et al. (1994) proved on a variable domain of immunoglobulin that it is possible to predict a stabilizing mutation with better than 60% success rate just by using a statistical method which determines the most frequent amino acid residue at a certain position of this domain. It was also suggested that this method would provide useful results not only for stabilization of variable domains of antibodies but also for domains of other proteins. However, it was never mentioned that this method could be extended to the entire protein. Furthermore, nothing is said about the program which was used to calculate the frequency of amino acid residues at a distinct position or whether scoring matrices were used as in the present case.

[0016] DNA sequences can be constructed starting from genomic or cDNA sequences coding for proteins, e.g. phytases known in the state of the art [for sequence information see references mentioned above, e.g. EP 684 313 or sequence data bases, for example like Genbank (Intelligenetics, California, USA), European Bioinformatics Institute (Hinston Hall, Cambridge, GB), NBRF (Georgetown University, Medical Centre, Washington DC, USA) and Vecbase (University of Wisconsin, Biotechnology Centre, Madison, Wisconsin. USA) or disclosed in the figures by methods of in vitro mutagenesis [see e.g. Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory Press, New York]. A widely used strategy for such "site directed mutagenesis", as originally outlined by Hurchinson and Edgell [J. Virol. 8, 181 (1971)], involves the annealing of a synthetic oligonucleotide carrying the desired nucleotide substitution to a target region of a single-stranded DNA sequence wherein the mutation should be introduced [for review see Smith, Annu. Rev. Genet. 19, 423 (1985) and for improved methods see references 2-6 in Stanssen et al., Nucl. Acid Res., 17, 4441-4454 (1989)]. Another possibility of mutating a given DNA sequence which is also preferred for the practice of the present invention is the mutagenesis by using the polymerase chain reaction (PCR). DNA as starting material can be isolated by methods known in the art and described e.g. in Sambrook et al. (Molecular Cloning) from the respective strains. For strain information see, e.g. EP 684 313 or any depository authority indicated below. Aspergillus niger [ATCC 9142], Myceliophthora thermophila [ATCC 48102], Talaromyces thermophilus [ATCC 20186] and Aspergillus fumigatus [ATCC 34625] have been redeposited according to the conditions of the Budapest Treaty at the American Type Culture Cell Collection under the following accession numbers: ATCC 74337, ATCC 74340, ATCC 74338 and ATCC 74339, respectively. It is however, understood that DNA encoding a consensus protein in accordance with the present invention can also be prepared in a synthetic manner as described, e.g. in EP 747 483 or the examples by methods known in the art.

[0017] Once complete DNA sequences have been obtained they can be integrated into vectors by methods known in the art and described e.g. in Sambrook et al. (s.a.) to overexpress the encoded polypeptide in appropriate host systems. However, a man skilled in the art knows that also the DNA sequences themselves can be used to transform the suitable host systems of the invention to get overexpression of the encoded polypeptide. Appropriate host systems

are for example fungi, like Aspergilli, e.g. Aspergillus niger [ATCC 9142] or Aspergillus ficuum [NRRL 3135] or like Trichoderma, e.g. Trichoderma reesei or yeasts, like Saccharomyces, e.g. Saccharomyces cerevisiae or Pichia, like Pichia pastoris, or Hansenula polymorpha, e.g. H. polymorpha (DSM5215) or plants, as described, e.g. by Pen et al., Bio/Technology 11, 811-814 (1994). A man skilled in the art knows that such microorganisms are available from depository authorities, e.g. the American Type Culture Collection (ATCC), the Centraalbureau voor Schimmelcultures (CBS) or the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSM) or any other depository authority as listed in the Journal "Industrial Property" [(1991) 1, pages 29-40]. Bacteria which can be used are e.g. E. coli, Bacilli as, e.g. Bacillus subtilis or Streptomyces, e.g. Streptomyces lividans (see e.g. Anné and Mallaert in FEMS Microbiol. Letters 114, 121 (1993). E. coli, which could be used are E. coli K12 strains e.g. M15 [described as DZ 291 by Villarejo et al. in J. Bacteriol. 120, 466-474 (1974)], HB 101 [ATCC No. 33694] or E. coli SG13009 [Gottesman et al., J. Bacteriol. 148, 265-273 (1981)].

[0018] Vectors which can be used for expression in fungi are known in the art and described e.g. in EP 420 358, or by Cullen et al. [Bio/Technology 5, 369-376 (1987)] or Ward in Molecular Industrial Mycology, Systems and Applications for Filamentous Fungi, Marcel Dekker, New York (1991), Upshall et al. [Bio/Technology 5, 1301-1304 (1987)] Gwynne et al. [Bio/Technology 5, 71-79 (1987)], Punt et al. [J. Biotechnol. 17, 19-34 (1991)] and for yeast by Sreekrishna et al. [J. Basic Microbiol. 28, 265-278 (1988), Biochemistry 28, 4117-4125 (1989)], Hitzemann et al. [Nature 293, 717-722 (1981)] or in EP 183 070, EP 183 071, EP 248 227, EP 263 311. Suitable vectors which can be used for expression in E. coli are mentioned, e.g. by Sambrook et al. [s.a.] or by Fiers et al. in Procd. 8th Int. Biotechnology Symposium" [Soc. Franc. de Microbiol., Paris (Durand et al., eds.), pp. 680-697 (1988)] or by Bujard et al. in Methods in Enzymology, eds. Wu and Grossmann, Academic Press, Inc. VoL 155, 416-433 (1987) and Stüber et al. in Immunological Methods, eds. Lefkovits and Pernis, Academic Press, Inc.. Vol. IV, 121-152 (1990). Vectors which could be used for expression in Bacilli are known in the art and described, e.g. in EP 405 370, Procd. Natl. Acad. Sci. USA 81, 439 (1984) by Yansura and Henner, Meth. Enzymol. 185, 199-228 (1990) or EP 207 459. Vectors which can be used for the expression in H. Polymorpha are known in the art and described, e.g. in Gellissen et al., Biotechnology 9, 291-295 (1991).

[0019] Either such vectors already carry regulatory elements, e.g. promotors, or the DNA sequences can be engineered to contain such elements. Suitable promotor elements which can be used are known in the art and are, e.g. for Trichoderma reesei the cbh1- [Haarki et al., Biotechnology 7, 596-600 (1989)] or the pki1-promotor [Schindler et al., Gene 130, 271-275 (1993)], for Aspergillus oryzae the amy-promotor [Christensen et al., Abstr. 19th Lunteren Lectures on Molecular Genetics F23 (1987), Christensen et al., Biotechnology 6, 1419-1422 (1988), Tada et al., Mol. Gen. Genet. 229, 301 (1991)], for Aspergillus niger the glaA- [Cullen et al., Bio/Technology 5, 369-376 (1987), Gwynne et al., Bio/Technology 5, 713-719 (1987), Ward in Molecular Industrial Mycology, Systems and Applications for Filamentous Fungi, Marcel Dekker, New York, 83-106 (1991)], alcA- [Gwynne et al., Bio/Technology 5, 718-719 (1987)], suc1- [Boddy et al., Curr. Genet. 24, 60-66 (1993)], aphA- [MacRae et al., Gene 71, 339-348 (1988), MacRae et al., Gene 132, 193-198 (1993)], tpiA-[McKnight et al., Cell 46, 143-147 (1986), Upshall et al., Bio/Technology 5, 1301-1304 (1987)], gpdA- [Punt et al., Gene 69, 49-57 (1988), Punt et al., J. Biotechnol. 17, 19-37 (1991)] and the pkiA-promotor [de Graaff et al., Curr. Genet. 22, 21-27 (1992)]. Suitable promotor elements which could be used for expression in yeast are known in the art and are, e.g. the pho5-promotor [Vogel et al., Mol. Cell. Biol., 2050-2057 (1989); Rudolf and Hinnen, Proc. Natl. Acad. Sci. 84, 1340-1344 (1987)] or the gap-promotor for expression in Saccharomyces cerevisiae and for Pichia pastoris, e.g. the aox1-promotor [Koutz et al., Yeast 5, 167-177 (1989); Sreekrishna et al., J. Basic Microbiol. 28, 265-278 (1988)], or the FMD promoter [Hollenberg et al., EPA No. 0299108] or MOX-promotor [Ledeboer et al., Nucleic Acids Res. 13, 3063-3082 (1985)] for H. polymorpha.

[0020] Once the consensus DNA sequence coding for the amino acid sequence of Fig 2 has been expressed in an appropriate host cell in a suitable medium the encoded protein can be isolated either from the medium in the case the protein is secreted into the medium or from the host organism in case such protein is present intracellularly by methods known in the art of protein purification or described in case of a phytase, e.g. in EP 420 358.

[0021] Once obtained the polypeptide of Fig 2 of the present invention can be characterized regarding their properties which make them useful in agriculture any assay known in the art and described e.g. by Simons et al. [Br. J. Nutr. 64, 525-540 (1990)], Schöner et al. [J. Anim. Physiol. a. Anim. Nutr. 66, 248-255 (1991)], Vogt [Arch. Geflügelk. 56, 93-98 (1992)], Jongbloed et al. [J. Anim. Sci., 70, 1159-1168 (1992)], Perney et al. [Poultry Sci. 72, 2106-2114 (1993)], Farrell et al., [J. Anim. Physiol. a. Anim. Nutr. 69, 278-283 (1993), Broz et al., [Br. Poultry Sci. 35, 273-280 (1994)] and Düngelhoef et al. [Animal Feed Sci. TechnoL 49, 1-10 (1994)] can be used.

[0022] In general the polypeptide of the present invention can be used without being limited to a specific field of application, e.g. in case of phytases for the conversion of inositol polyphosphates, like phytate to inositol and inorganic phosphate.

[0023] Furthermore the polypeptide of the present invention can be used in a process for the preparation of a pharmaceutical composition or compound food or feeds wherein the components of such a composition are mixed with one or more polypeptides of the present invention. Accordingly compound food or feeds or pharmaceutical compositions comprising one or more polypeptides of the present invention are also an object of the present invention. A man skilled

in the art is familiar with their process of preparation. Such pharmaceutical compositions or compound foods or feeds can further comprise additives or components generally used for such purpose and known in the state of the art.

[0024] Before describing the present invention in more detail a short explanation of the Tables and enclosed Figures is given below.

Table 1: Vote weights of the amino acid sequences of the fungal phytases used. The table shows the vote weights used to calculate the consensus sequence of the fungal phytases.

Table 2: Homology of the fungal phytases. The amino acid sequences of the phytases used in the alignment were compared by the program GAP (GCG program package, 9; Devereux *et al.*, 1984) using the standard parameters. The comparison was restricted to the part of the sequence that was also used for the alignment (see legend to Figure 1) lacking the signal peptide which was ratherdivergent. The numbers above and beneath the diagonal represent the amino acid identities and similarities, respectively.

Table 3: Homology of the amino acid sequence of fungal consensus phytase to the phytases used for its calculation. The amino acid sequences of all phytases were compared with the fungal consensus phytase sequence using the program GAP (GCG program package, 9.0). Again, the comparison was restricted to that part of the sequence that was used in the alignment.

Table 4: Primers used for the introduction of single mutations into fungal consensus phytase. For the introduction of each mutation, two primers containing the desired mutation were required (see Example 8). The changed triplets are highlighted in bold letters.

Table 5: Temperature optimum and T_m-value of fungal consensus phytase and of the phytases from *A. fumigatus*, *A. niger*, *A. nidulans*, and *M. thermophila*. The temperature optima were taken from Figure 3. [a] The T_m-values were determined by differential scanning calorimetry as described in Example 10 and shown in Figure 7.

Figure 1: Calculation of the consensus phytase sequence from the alignment of nearly all known fungal phytase amino acid sequences. The letters represent the amino acid residues in the one-letter code. The following sequences were used for the alignment: *phyA* from *Aspergillus terreus* 9A-1 (Mitchell *et al.*, 1997; from amino acid (aa) 27), *phyA* from *Aspergillus terreus* cbs116.46 (van Loon *et al.*, 1997; from aa 27), *phyA* from *Aspergillus niger* var. *awamori* (Piddington *et al.*, 1993; from aa 27), *phyA* from *Aspergillus niger* T213; from aa 27), *phyA* from *Aspergillus niger* strain NRRL3135 (van Hartingsveldt *et al.*, 1993; from aa 27), *phyA* from *Aspergillus fumigatus* ATCC 13073 (Pasamontes *et al.*, 1997b; from aa 25), *phyA* from *Aspergillus fumigatus* ATCC 32722 (van Loon *et al.*, 1997; from aa 27), *phyA* from *Aspergillus fumigatus* ATCC 58128 (van Loon *et al.*, 1997; from aa 27), *phyA* from *Aspergillus fumigatus* ATCC 26906 (van Loon *et al.*, 1997; from aa 27), *phyA* from *Aspergillus fumigatus* ATCC 32239 (van Loon *et al.*, 1997; from aa 30), *phyA* from *Aspergillus nidulans* (Pasamontes *et al.*, 1997a; from aa 25), *phyA* from *Talaromyces thermophilus* (Pasamontes *et al.*, 1997a; from aa 24), and *phyA* from *Myceliophthora thermophila* (Mitchell *et al.*, 1997; from aa 19). The alignment was calculated using the program PILEUP. The location of the gaps was refined by hand. Capitalized amino acid residues in the alignment at a given position belong to the amino acid coalition that establish the consensus residue. In bold, beneath the calculated consensus sequence, the amino acid sequence of the finally constructed fungal consensus phytase (Fcp) is shown. The gaps in the calculated consensus sequence were filled by hand according to principals stated in Example 2.

Figure 2: DNA sequence of the fungal consensus phytase gene (*fcp*) and of the primers synthesized for gene construction. The calculated amino acid sequence (Figure 1) was converted into a DNA sequence using the program BACKTRANSLATE (Devereux *et al.*, 1984) and the codon frequency table of highly expressed yeast genes (GCG program package, 9.0). The signal peptide of the phytase from *A. terreus* cbs was fused to the *N*-terminus. The bold bases represent the sequences of the oligonucleotides used to generate the gene. The names of the respective oligonucleotides are noted above or below the sequence. The underlined bases represent the start and stop codon of the gene. The bases written in italics show the two introduced *Eco* RI sites.

Figure 3: Temperature optimum of fungal consensus phytase and other phytases used to calculate the consensus sequence. For the determination of the temperature optimum, the phytase standard assay was performed at a series of temperatures between 37 and 85 °C. The phytases used were purified according to Example 5. ▽, fungal consensus phytase; ▼, *A. fumigatus* 13073 phytase; □, *A. niger* NRRL3135 phytase; ○, A. *nidulans* phytase; ■, *A. terreus* 9A-1 phytase; •, *A. terreus* cbs phytase.

Figure 4: The pH-dependent activity profile of fungal consensus phytase and of the mutant Q50L, Q50T, and Q50G. The phytase activity was determined using the standard assay in appropriate buffers (see Example 9) at different pH-values. Plot a) shows a comparison of fungal consensus phytase (●) to the mutants Q50L (▽), Q50T (▼), and Q50G (○) in percent activity. Plot b) shows a comparison of fungal consensus phytase (○) to mutant Q50L (●) and Q50T (▽) using the specific activity of the purified enzymes expressed in *H. polymorpha.*

Figure 5: The pH-dependent activity profile of the mutants Q50L, Y51N and Q50T, Y51N in comparison to the mutants Q50T and Q50L of fungal consensus phytase. The phytase activity was determined using the standard assay in appropriate buffers (see Example 9) at different pH-values. Graph a) shows the influence of the mutation Y51N (●) on mutant Q50L (○). Graph b) shows the influence of the same mutation (●) on mutant Q50T (○).

Figure 6: Substrate specificity of fungal consensus phytase and its mutants Q50L, Q50T, and Q50G. The bars represent the relative activity in comparison to the activity with phytic acid (100%) with a variety of known natural and synthetic phosphorylated compounds.

Figure 7: Differential scanning calorimetry (DSC) of fungal consensus phytase and its mutant Q50T. The protein samples were concentrated to ca. 50-60 mg/ml and extensively dialyzed against 10 mM sodium acetate, pH 5.0. A constant heating rate of 10 °C/min was applied up to 90 °C. DSC of consensus phytase Q50T (upper graph) yielded in a melting temperature of 78.9 °C, which is nearly identical to the melting point of fungal consensus phytase (78.1 °C, lower graph).

Examples

Reference Example

Homology Modeling of *A. fumigatus* and *A. terreus* cbs116.46 phytase

[0025] The amino acid sequences of *A. fumigatus* and *A. terreus* cbs116.46 phytase were compared with the sequence of *A. niger* NRRL 3135 phytase (see Figure 1) for which the three-dimensional structure had been determined by X-ray crystallography.

[0026] A multiple amino acid sequence alignment of *A. niger* NRRL 3135 phytase, *A. fumigatus* phytase and *A. terreus* cbs116.46 phytase was calculated with the program "PILEUP" (Prog. Menu for the Wisconsin Package, version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison Wisconcin, USA 53711). The three-dimensional models of *A. fumigatus* phytase and *A. terreus* cbs116.46 phytase were built by using the structure of *A. niger* NRRL 3135 phytase as template and exchanging the amino acids of *A. niger* NRRL 3135 phytase according to the sequence alignment to amino acids of *A. fumigatus* and *A. terreus* cbs116.46 phytases, respectively. Model construction and energy optimization were performed by using the program Moloc (Gerber and Müller, 1995). C-alpha positions were kept fixed except for new insertions/deletions and in loop regions distant from the active site.

[0027] Only small differences of the modelled structures to the original crystal structure could be observed in external loops. Furthermore the different substrate molecules that mainly occur on the degradation pathway of phatic acid (*myo*-inositol-hexakisphosphate) by *Pseudomonas sp. bacterium* phytase and, as far as determined, by *A. niger* NRRL 3135 phytase (Cosgrove, 1980) were constructed and forged into the active site cavity of each phytase structure. Each of these substrates was oriented in a hypothetical binding mode proposed for histidine acid phosphatases (Van Etten, 1982). The scissile phosphate group was oriented towards the catalytically essential His 59 to form the covalent phosphoenzyme intermediate. The oxygen of the substrate phosphoester bond which will be protonated by Asp 339 after cleavage was orientated towards the proton donor. Conformational relaxation of the remaining structural part of the substrates as well as the surrounding active site residues was performed by energy optimization with the program Moloc.

[0028] Based on the structure models the residues pointing into the active site cavity were identified. More than half (60%) of these positions were identical between these three phytases, whereas only few positions were not conserved (see Figure 1). This observation could be extended to four additional phytase sequences (*A. nidulans, A. terreus* 9A1, *Talaromyces thermophilus, Myceliophthora thermophila*).

Example 1

Alignment of the amino acid sequence of the fungal phytases

[0029] The alignment was calculated using the program PILEUP from the Sequence Analysis Package Release 9.0

(Devereux *et al.*, 1984) with the standard parameter (gap creation penalty 12, gap extension penalty 4). The location of the gaps was refined using a text editor. The following sequences (see Figure 1) without the signal sequence were used for the performance of the alignment starting with the amino acid (aa) mentioned below:

phyA gene from *Aspergillus terreus* 9A-1, aa 27 (Mitchell *et al.*, 1997)
phyA gene from *Aspergillus terreus* cbs116.46, aa 27 (van Loon *et al.*, 1997)
phyA gene from *Aspergillus niger* var. *awamori*, aa 27 (Piddington *et al.*, 1993)
phyA gene from *Aspergillus niger* T213, aa 27
phyA gene from *Aspergillus niger* strain NRRL3135, aa 27 (van Hartingaveldt *et al.*, 1993)
phyA gene from *Aspergillus fumigatus* ATCC 13073, aa 26 (Pasamontes *et al.*, 1997)
phyA gene from *Aspergillus fumigatus* ATCC 32722, aa 26 (van Loon *et al.*, 1997)
phyA gene from *Aspergillus fumigatus* ATCC 58128, aa 26 (van Loon *et al.*, 1997)
phyA gene from *Aspergillus fumigatus* ATCC 26906, aa 26 (van Loon *et al.*, 1997)
phyA gene from *Aspergillus fumigatus* ATCC 32239, aa 30 (van Loon *et al.*, 1997)
phyA gene from *Aspergillus nidulans*, aa 25 (Roche Nr. R1288, Pasamontes *et al.*, 1997a)
phyA gene from *Talaromyces thermophilus* ATCC 20186, aa 24 (Pasamontes *et al.*, 1997a)
phyA gene from *Myceliophthora thermophila*, aa 19 (Mitchell *et al.*, 1997)

Table 2 shows the homology of the phytase sequences mentioned above.

Example 2

Calculation of the amino acid sequence of fungal consensus phytases

[0030] Using the refined alignment of Example 1 as input, the consensus sequence was calculated by the program PRETTY from the Sequence Analysis Package Release 9.0 (Devereux *et al.*, 1984). PRETTY prints sequences with their columns aligned and can display a consensus sequence for the alignment. A vote weight that pays regard to the similarity between the amino acid sequences of the phytases aligned were assigned to all sequences. The vote weight was set such as the combined impact of all phytases from one sequence subgroup (same species of origin but different strains), e. g. the amino acid sequences of all phytases from *A. fumigatus*, on the election was set one, that means that each sequence contributes with a value of 1 divided by the number of strain sequences (see Table 1). By this means, it was possible to prevent that very similar amino acid sequences, e. g. of the phytases from different *A. fumigatus* strains, dominate the calculated consensus sequence.

[0031] The program PRETTY was started with the following parameters: The plurality defining the number of votes below which there is no consensus was set on 2.0. The threshold, which determines the scoring matrix value below which an amino acid residue may not vote for a coalition of residues, was set on 2. PRETTY used the PrettyPep.Cmp consensus scoring matrix for peptides.

[0032] Ten positions of the alignment (position 46, 66, 82, 138, 162, 236, 276, 279, 280, 308; Figure 1), for which the program was not able to determine a consensus residue, were filled by hand according to the following rules: if a most frequent residue existed, this residue was chosen (138, 236, 280); if a prevalent group of chemically similar or equivalent residues occurred, the most frequent or, if not available, one residues of this group was selected (46, 66, 82, 162, 276, 308). If there was either a prevalent residue nor a prevalent group, one of the occurring residues was chosen according to common assumption on their influence on the protein stability (279). Eight other positions (132, 170, 204, 211, 275, 317, 384, 447; Figure 1) were not filled with the amino acid residue selected by the program but normally with amino acids that occur with the same frequency as the residues that were chosen by the program. In most cases, the slight underrating of the three *A. niger* sequences (sum of the vote weights: 0.99) was eliminated by this corrections.

[0033] Table 3 shows the homology of the calculated fungal consensus phytase amino acid sequence to the phytase sequences used for the calculation.

Example 3

Conversion of the fungal consensus phytase amino acid sequence to a DNA sequence

[0034] The first 26 amino acid residues of *A. terreus* cbs116.46 phytase were used as signal peptide and, therefore, fused to the N-terminus of all consensus phytases. For this stretch, we used a special method to calculate the corresponding DNA sequence. Purvis *et al.* (1987) proposed that the incorporation of rare codons in a gene has an influence on the folding efficiency of the protein. Therefore, at least the distribution of rare codons in the signal sequence of *A.*

terreus cbs116.46, which was used for the fungal consensus phytase and which is very important for secretion of the protein, but converted into the *S. cerevisiae* codon usage, was transferred into the new signal sequence generated for expression in *S. cerevisiae*. For the remaining parts of the protein, we used the codon frequency table of highly expressed *S. cerevisiae* genes, obtained from the GCG program package, to translate the calculated amino acid sequence into a DNA sequence.

[0035] The resulting sequence of the *fcp* gene are shown in Figure 2.

Example 4

Construction and cloning of the fungal consensus phytase genes

[0036] The calculated DNA sequence of fungal consensus phytase was divided into oligonucleotides of 85 bp, alternately using the sequence of the sense and the anti-sense strand. Every oligonucleotide overlaps 20 bp with its previous and its following oligonucleotide of the opposite strand. The location of all primers, purchased by Microsynth, Balgach (Switzerland) and obtained in a PAGE-purified form, is indicated in Figure 2.

[0037] In three PCR reactions, the synthesized oligonucleotides were composed to the entire gene. For the PCR, the High Fidelity Kit from Boehringer Mannheim (Boehringer Mannheim, Mannheim, Germany) and the thermo cycler The Protokol™ from AMS Biotechnology (Europe) Ltd. (Lugano, Switzerland) were used.

[0038] Oligonucleotide CP-1 to CP-10 (Mix 1, Figure 2) were mixed to a concentration of 0.2 pMol/µl per each oligonucleotide. A second oligonucleotide mixture (Mix 2) was prepared with CP-9 to CP-22 (0.2 pMol/µl per each oligonucleotide). Additionally, four short primers were used in the PCR reactions:

CP-a: *Eco* RI
5'-TAT AT<u>G AAT TC</u>A TGG GCG TGT TCG TC-3'

CP-b:
5'-TGA AAA GTT CAT TGA AGG TTT C-3'

CP-c:
5'-TCT TCG AAA GCA GTA CAA GTA C-3'

CP-e: *Eco* RI
5'-TAT AT<u>G AAT TC</u>T TAA GCG AAA C-3'

PCR reaction *a*:	10 µl Mix 1 (2.0 pmol of each oligonucleotide)
	2 µl nucleotides (10 mM each nucleotide)
	2 µl primer CP-a (10 pmol/µl)
	2 µl primer CP-c (10 pmol/µl)
	10,0 µl PCR buffer
	0.75 µl polymerase mixture
	73.25 µl H_2O
PCR reaction *b*:	10 µl Mix 2 (2.0 pmol of each oligonucleotide)
	2 µl nucleotides (10 mM each nucleotide)
	2 µl primer CP-b (10 pmol/µl)
	2 µl primer CP-e (10 pmol/µl)
	10,0 µl PCR buffer
	0.75 µl polymerase mixture (2.6 U)

73.25 µl H_2O

Reaction conditions for PCR reaction *a* and *b*:

step 1	2 min - 45°C
step 2	30 sec - 72°C
step 3	30 sec - 94°C
step 4	30 sec - 52°C
step 5	1 min - 72°C

Step 3 to 5 were repeated 40-times.

[0039] The PCR products (670 and 905 bp) were purified by an agarose gel electrophoresis (0.9% agarose) and a following gel extraction (QIAEX II Gel Extraction Kit, Qiagen, Hilden, Germany). The purified DNA fragments were used for the PCR reaction c.

PCR reaction *c*:

6 µl PCR product of reaction a (≈50 ng)
6 µl PCR product of reaction b (≈50 ng)
2 µl primer CP-a (10 pmol/µl)
2 µl primer CP-e (10 pmol/µl)
10,0 µl PCR buffer
0.75 µl polymerase mixture (2.6 U)
73.25 µl H_2O

Reaction conditions for PCR reaction *c*:

step 1	2 min - 94°C
step 2	30 sec - 94°C
step 3	30 sec - 55°C
step 4	1 min - 72°C

Step 2 to 4 were repeated 31-times.

[0040] The resulting PCR product (1.4 kb) was purified as mentioned above, digested with *Eco* RI, and ligated in an *Eco* RI-digested and dephosphorylated pBsk(-)-vector (Stratagene, La Jolla, CA, USA). 1 µl of the ligation mixture was used to transform *E. coli* XL-1 competent cells (Stratagene, La Jolla, CA, USA). All standard procedures were carried out as described by Sambrook *et al.* (1987). The constructed fungal consensus phytase gene (*fcp*) was verified by sequencing (plasmid pBsk-fcp).

Example 5

Expression of the fungal consensus phytase gene *fcp* and its variants in *Saccharomyces cerevisiae* and their purification from culture supernatant

[0041] A fungal consensus phytase gene was isolated from the plasmid pBsk-*fcp* ligated into the *Eco* RI sites of the expression cassette of the *Saccharomyces cerevisiae* expression vector pYES2 (Invitrogen, San Diego, CA, USA) or subcloned between the shortened GAPFL (glyceraldhyde-3-phosphate dehydrogenase) promoter and the *pho5* terminator as described by Janes *et al.* (1990). The correct orientation of the gene was checked by PCR. Transformation of *S. cerevisiae* strains. e. g. INVSc1 (Invitrogen, San Diego, CA, USA) was done according to Hinnen *et al.* (1978). Single colonies harboring the phytase gene under the control of the GAPFL promoter were picked and cultivated in 5 ml selection medium (SD-uracil, Sherman *et al.*, 1986) at 30°C under vigorous shaking (250 rpm) for one day. The preculture was then added to 500 ml YPD medium (Sherman *et al.*, 1986) and grown under the same conditions. Induction of the *gal1* promoter was done according to manufacturer's instruction. After four days of incubation cell broth was centrifuged (7000 rpm, GS3 rotor, 15 min, 5°C) to remove the cells and the supernatant was concentrated by way

of ultrafiltration in Amicon 8400 cells (PM30 membranes) and ultrafree-15 centrifugal filter devices (Biomax-30K, Millipore, Bedford, MA, USA). The concentrate (10 ml) was desalted on a 40 ml Sephadex G25 Superfine column (Pharmacia Biotech, Freiburg, Germany), with 10 mM sodium acetate, pH 5.0, serving as elution buffer. The desalted sample was brought to 2 M $(NH_4)_2SO_4$ and directly loaded onto a 1 ml Butyl Sepharose 4 Fast Flow hydrophobic interaction chromatography column (Pharmacia Biotech, Feiburg, Germany) which was eluted with a linear gradient from 2 M to 0 M $(NH4)_2SO_4$ in 10 mM sodium acetate, pH 5.0. Phytase was eluted in the break-through, concentrated and loaded on a 120 ml Sephacryl S-300 gel permeation chromatography column (Pharmacia Biotech, Freiburg, Germany). Fungal consensus phytase and fungal consensus phytase 7 eluted as a homogeneous symmetrical peak and was shown by SDS-PAGE to be approx. 95% pure.

Example 6

Expression of the fungal consensus phytase genes *fcp* and its variants in *Hansenula polymorpha*

[0042] The phytase expression vectors, used to transform *H. polymorpha,* was constructed by inserting the *Eco* RI fragment of pBsk-*fcp* encoding the consensus phytase or a variant into the multiple cloning site of the *H. polymorpha* expression vector pFPMT121, which is based on an *ura3* selection marker and the *FMD* promoter. The 5' end of the *fcp* gene is fused to the *FMD* promoter, the 3' end to the *MOX* terminator (Gellissen *et al.*, 1996; EP 0299 108 B). The resulting expression vector are designated pFPMT*fcp* and pBsk⁻ *fcp*7.

[0043] The constructed plasmids were propagated in *E. coli.* Plasmid DNA was purified using standard state of the art procedures. The expression plasmids were transformed into the *H. polymorpha* strain RP11 deficient in orotidine-5'-phosphate decarboxylase (*ura3*) using the procedure for preparation of competent cells and for transformation of yeast as described in Gelissen *et al.* (1996). Each transformation mixture was plated on YNB (0.14% w/v Difco YNB and 0.5% ammonium sulfate) containing 2% glucose and 1.8% agar and incubated at 37 °C. After 4 to 5 days individual transformant colonies were picked and grown in the liquid medium described above for 2 days at 37 °C. Subsequently, an aliquot of this culture was used to inoculate fresh vials with YNB-medium containing 2% glucose. After seven further passages in selective medium, the expression vector integrates into the yeast genome in multimeric form. Subsequently, mitotically stable transformants were obtained by two additional cultivation steps in 3 ml non-selective liquid medium (YPD, 2% glucose, 10 g yeast extract, and 20 g peptone). In order to obtain genetically homogeneous recombinant strains an aliquot from the last stabilisation culture was plated on a selective plate. Single colonies were isolated for analysis of phytase expression in YNB containing 2% glycerol instead of glucose to derepress the *fmd* promoter. Purification of the fungal consensus phytases was done as described in Example 5.

Example 7

Expression of the fungal consensus genes *fcp* and its variants in *Aspergillus niger*

[0044] Plasmid pBsk⁻*fcp* or the corresponding plasmid of a variant of the *fcp* gene were used as template for the introduction of a *Bsp* HI-site upstream of the start codon of the genes and an *Eco* RV-site downstream of the stop codon. The Expand™ High Fidelity PCR Kit (Boehringer Mannheim, Mannheim, Germany) was used with the following primers:

Primer Asp-1:

***Bsp* HI**

5'-TAT ATC ATG AGC GTG TTC GTC GTG CTA CTG TTC-3'

Primer Asp-2 for cloning of *fcp* and *fcp7*:

3'-ACC CGA CTT ACA AAG CGA ATT CTA TAG ATA TAT-5'

***Eco* RV**

[0045] The reaction was performed as described by the supplier. The PCR-amplified *fcp* gene had a new *Bsp* HI site

at the start codon, introduced by primer Asp-1, which resulted in a replacement of the second amino acid residue glycine by serine. Subsequently, the DNA-fragment was digested with *Bsp* HI and *Eco* RV and ligated into the *Nco* I site downstream of the glucoamylase promoter of *Aspergillus niger* (*glaA*) and the *Eco* RV site upstream of the *Aspergillus nidulans* tryptophan C terminator (*trpC*) (Mullaney *et al.*, 1985). After this cloning step, the genes were sequenced to detect possible failures introduced by PCR. The resulting expression plasmids which basically corresponds to the pGLAC vector as described in Example 9 of EP 684 313, contained the orotidine-5'-phosphate decarboxylase gene (*pyr4*) of *Neurospora crassa* as a selection marker.Transformation of *Aspergillus niger* and expression of the consensus phytase genes was done as described in EP 684 313. The fungal consensus phytases were purified as described in Example 5.

Example 8

Construction of muteins of fungal consensus phytase

[0046] To construct muteins for expression in *A. niger, S. cerevisiae,* or *H. polymorpha,* the corresponding expression plasmid containing the fungal consensus phytase gene was used as template for site-directed mutagenesis. Mutations were introduced using the "quick exchange™ site-directed mutagenesis kit" from Stratagene (La Jolla, CA, USA) following the manufacturer's protocol and using the corresponding primers. All mutations made and the corresponding primers are summarized in Table 4. Clones harboring the desired mutation were identified by DNA sequence analysis as known in the art. The mutated phytase were verified by sequencing of the complete gene.

Example 9

Determination of the phytase activity and of the temperature optimum of the consensus phytase and its variants

[0047] Phytase activity was determined basically as described by Mitchell *et al.* (1997). The activity was measured in a assay mixture containing 0.5% phytic acid (=5 mM), 200 mM sodium acetate, pH 5.0. After 15 min incubation at 37 °C, the reaction was stopped by addition of an equal volume of 15% trichloroacetic acid. The liberated phosphate was quantified by mixing 100 µl of the assay mixture with 900 µl H_2O and 1 ml Of 0.6 M H_2SO_4, 2% ascorbic acid and 0.5% ammonium molybdate. Standard solutions of potassium phosphate were used as reference. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol phosphate per minute at 37 °C. The protein concentration was determined using the enzyme extinction coefficient at 280 nm calculated according to Pace *et al.* (1995): fungal consensus phytase, 1.101; fungal consensus phytase 7, 1.068.

[0048] In case of pH-optimum curves, purified enzymes were diluted in 10 mM sodium acetate, pH 5.0. Incubations were started by mixing aliquots of the diluted protein with an equal volume of 1% phytic acid (≈10 mM) in a series of different buffers: 0.4 M glycine/HCl, pH 2.5; 0.4 M acetate/NaOH, pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5; 0.4 M imidazole/HCl, pH 6.0, 6.5; 0.4 M Tris/HCl pH 7.0, 7.5, 8.0, 8.5, 9.0. Control experiments showed that pH was only slightly affected by the mixing step. Incubations were performed for 15 min at 37 °C as described above.

[0049] For determination of the substrate specificities of the phytases, phytic acid in the assay mixture was replaced by 5 mM concentrations of the respective phosphate compounds. The activity tests were performed as described above.

[0050] For determination of the temperature optimum, enzyme (100 µl) and substrate solution (100 µl) were preincubated for 5 min at the given temperature. The reaction was started by addition of the substrate solution to the enzyme. After 15 min incubation, the reaction was stopped with trichloroacetic acid and the amount of phosphate released was determined.

[0051] The pH-optimum of the original fungal consensus phytase was around pH 6.0-6.5 (70 U/mg). By introduction of the Q50T mutation, the pH-optimum shifted to pH 6.0 (130 U/mg), while the replacement by a leucine at the same position resulted in a maximum activity around pH 5.5 (212 U/mg). The exchange Q50G resulted in a pH-optimum of the activity above pH 6.0 (see Figure 4). The exchange of tyrosine at position 51 with asparagine resulted in a relative increase of the activity below pH 5.0 (see Figure 5). Especially by the Q50L mutation, the specificity for phytate of fungal consensus phytase was drastically increased (see Figure 6).

[0052] The temperature optimum of fungal consensus phytase (70 °C) was 15-25 °C higher than the temperature optimum of the wild-type phytases (45-55 °C) which were used to calculate the consensus sequence (see Table 5 and Figure 3).

Example 10

Determination of the melting point by differential scanning calorimetry (DSC)

[0053] In order to determine the unfolding temperature of the fungal consensus phytases, differential scanning calorimetry was applied as previously published by Brugger *et al.* (1997). Solutions of 50-60 mg/ml homogeneous phytase were used for the tests. A constant heating rate of 10 °C/min was applied up to 90 °C.

[0054] The determined melting points clearly show the strongly improved thermostability of the fungal consensus phytase in comparison to the wild-type phytases (see Table 5 and Figure 7). Figure 7 shows the melting profile of fungal consensus phytase and its mutant Q50T. Its common melting point was determined between 78 to 79 °C.

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[0055]

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Table 1

Aspergillus terreus 9A-1 phytase	0.50
Aspergillus terreus cbs116.46 phytase	0.50
Aspergillus niger var. *awamori* phytase	0.3333
Aspergillus niger T213 phytase	0.3333
Aspergillus niger NRRL3135 phytase	0.3333
Aspergillus fumigatus ATCC 13073 phytase	0.20
Aspergillus fumigatus ATCC 32722 phytase	0.20
Aspergillus fumigatus ATCC 58128 phytase	0.20
Aspergillus fumigatus ATCC 26906 phytase	0.20
Aspergillus fumigatus ATCC 32239 phytase	0.20
Aspergillus nidulans phytase	1.00
Talaromyces thermophilus ATCC 20186 phytase	1.00
Myceliophthora thermophila phytase	1.00

Table 2

% identity

	A. terreus 9A-1	*A. terreus* cbs116.46	*A. niger* NRRL 3135	*A. fumigatus* 13073	*A. nidulans*	*T. thermophilus*	*M. thermophila*
A. terreus 9A-1		89.1	62.0	60.6	59.3	58.3	48.6
A. terreus cbs	90.7		63.6	62.0	61.2	59.7	49.1
A. niger NRRL 3135	67.3	68.9		66.8	64.2	62.5	49.4
A. fumigatus 13073	66.1	67.2	71.1		68.0	62.6	53.0
A. nidulans	65.0	66.7	69.0	73.3		60.5	52.5
T. thermophilus	63.8	64.5	68.9	68.1	67.4		49.8
M. thermophila	53.7	54.6	57.6	61.0	59.9	57.8	

% similarity

Table 3:

Phytase	Identity [%]	Similarity [%]
A. niger T213	76.6	79.6
A. niger var. *awamori*	76.6	79.6
A. niger NRRL3135	76.6	79.4
A. nidulans	77.4	81.5
A. terreus 9A-1	70.7	74.8
A. terreus cbs116.46	72.1	75.9
A. fumigatus 13073	80.0	83.9

Table 3: (continued)

Phytase	Identity [%]	Similarity [%]
A. fumigatus 32239	78.2	82.3
T. thermophilus	72.7	76.8
M. thermophila	58.3	64.5

Table 4

mutation	Primer set
	Ssp BI
Q50L	5'-CAC TTG TGG GGT TTG TAC AGT CCA TAC TTC TC-3' 5'-GAG AAG TAT GGA CTG TAC AAA CCC CAC AAG TG-3'
	Kpn I
Q50T	5'-CAC TTG TGG GGT ACC TAC TCT CCA TAC TTC TC-3' 5'-GA GAA GTA TGG AGA GTA GGT ACC CCA CAA GTG-3'
Q50G	5'-CAC TTG TGG GGT GGT TAC TCT CCA TAC TTC TC-3' 5'-GA GAA GTA TGG AGA GTA ACC ACC CCA CAA GTG-3'
	Kpn I
Q50T-Y51N	5'-CAC TTG TGG GGT ACC AAC TCT CCA TAC TTC TC-3' 5'-GA GAA GTA TGG AGA GTT GGT ACC CCA CAA GTG-3'
	Bsa I
Q50L-Y51N	5'-CAC TTG TGG GGT CTC AAC TCT CCA TAC TTC TC-3' 5'-GA GAA GTA TGG AGA GTT GAG ACC CCA CAA GTG-3'

Table 5

phytase	temperature optimum	*Tm*[a]
Consensus phytase	70 °C	78.0 °C
A. niger NRRL3135	55 °C	63.3 °C
A. fumigatus 13073	55°C	62.5 °C
A. terreus 9A-1	49 °C	57.5 °C
A. terreus cbs	45 °C	58.5 °C

Table 5 (continued)

phytase	temperature optimum	Tm[a]
A. nidulans	45 °C	55.7 °C
M. thermophila	55 °C	-

SEQUENCE LISTING

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Asp Glu Ser Ala Ile Ser Pro Asp Val Pro Asp Asp Cys Arg Val Thr
        35                  40                  45
Phe Val Gln Val Leu Ser Arg His Gly Ala Arg Tyr Pro Thr Ser Ser
    50                  55                  60
Lys Ser Lys Ala Tyr Ser Ala Leu Ile Glu Ala Ile Gln Lys Asn Ala
65                  70                  75                  80
Thr Ala Phe Lys Gly Lys Tyr Ala Phe Leu Lys Thr Tyr Asn Tyr Thr
                85                  90                  95
Leu Gly Ala Asp Asp Leu Thr Pro Phe Gly Glu Asn Gln Met Val Asn
            100                 105                 110
Ser Gly Ile Lys Phe Tyr Arg Arg Tyr Lys Ala Leu Ala Arg Lys Ile
        115                 120                 125
Val Pro Phe Ile Arg Ala Ser Gly Ser Asp Arg Val Ile Ala Ser Ala
    130                 135                 140
Glu Lys Phe Ile Glu Gly Phe Gln Ser Ala Lys Leu Ala Asp Pro Gly
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Ser Gln Pro His Gln Ala Ser Pro Val Ile Asp Val Ile Ile Pro Glu
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Gly Ser Gly Tyr Asn Asn Thr Leu Asp His Gly Thr Cys Thr Ala Phe
            180                 185                 190
Glu Asp Ser Glu Leu Gly Asp Asp Val Glu Ala Asn Phe Thr Ala Leu
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Phe Ala Pro Ala Ile Arg Ala Arg Leu Glu Ala Asp Leu Pro Gly Val
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Thr Leu Thr Asp Glu Asp Val Val Tyr Leu Met Asp Met Cys Pro Phe
225                 230                 235                 240
Glu Thr Val Ala Arg Thr Ser Asp Ala Thr Glu Leu Ser Pro Phe Cys
                245                 250                 255
Ala Leu Phe Thr His Asp Glu Trp Arg Gln Tyr Asp Tyr Leu Gln Ser
            260                 265                 270
Leu Gly Lys Tyr Tyr Gly Tyr Gly Ala Gly Asn Pro Leu Gly Pro Ala
        275                 280                 285
Gln Gly Val Gly Phe Ala Asn Glu Leu Ile Ala Arg Leu Thr Arg Ser
    290                 295                 300

Pro Val Gln Asp His Thr Ser Thr Asn His Thr Leu Asp Ser Asn Pro
305                 310                 315                 320
Ala Thr Phe Pro Leu Asn Ala Thr Leu Tyr Ala Asp Phe Ser His Asp
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Asn Ser Met Ile Ser Ile Phe Phe Ala Leu Gly Leu Tyr Asn Gly Thr
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Ala Pro Leu Ser Thr Thr Ser Val Glu Ser Ile Glu Glu Thr Asp Gly
        355                 360                 365
Tyr Ser Ala Ser Trp Thr Val Pro Phe Gly Ala Arg Ala Tyr Val Glu
    370                 375                 380
Met Met Gln Cys Gln Ala Glu Lys Glu Pro Leu Val Arg Val Leu Val
385                 390                 395                 400
Asn Asp Arg Val Val Pro Leu His Gly Cys Ala Val Asp Lys Leu Gly
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Thr Val Asp Gly Gly Tyr Gln Cys Phe Pro Glu Ile Ser His Leu Trp
        35                  40                  45
Gly Gln Tyr Ser Pro Tyr Phe Ser Leu Glu Asp Glu Ser Ala Ile Ser
    50                  55                  60
Pro Asp Val Pro Asp Asp Cys Arg Val Thr Phe Val Gln Val Leu Ser
65                  70                  75                  80
Arg His Gly Ala Arg Tyr Pro Thr Ser Ser Lys Ser Lys Ala Tyr Ser
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Ala Leu Ile Glu Ala Ile Gln Lys Asn Ala Thr Ala Phe Lys Gly Lys
            100                 105                 110
Tyr Ala Phe Leu Lys Thr Tyr Asn Tyr Thr Leu Gly Ala Asp Asp Leu
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Ser Gly Ser Asp Arg Val Ile Ala Ser Ala Glu Lys Phe Ile Glu Gly
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Phe Gln Ser Ala Lys Leu Ala Asp Pro Gly Ser Gln Pro His Gln Ala
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Ser Pro Val Ile Asp Val Ile Ile Pro Glu Gly Ser Gly Tyr Asn Asn
        195                 200                 205
Thr Leu Asp His Gly Thr Cys Thr Ala Phe Glu Asp Ser Glu Leu Gly
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Asp Asp Val Glu Ala Asn Phe Thr Ala Leu Phe Ala Pro Ala Ile Arg
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Ala Arg Leu Glu Ala Asp Leu Pro Gly Val Thr Leu Thr Asp Glu Asp
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Val Val Tyr Leu Met Asp Met Cys Pro Phe Glu Thr Val Ala Arg Thr
            260                 265                 270
Ser Asp Ala Thr Glu Leu Ser Pro Phe Cys Ala Leu Phe Thr His Asp
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Ser Thr Asn His Thr Leu Asp Ser Asn Pro Ala Thr Phe Pro Leu Asn
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Ala Thr Leu Tyr Ala Asp Phe Ser His Asp Asn Ser Met Ile Ser Ile
        355                 360                 365
Phe Phe Ala Leu Gly Leu Tyr Asn Gly Thr Ala Pro Leu Ser Thr Thr
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Ser Val Glu Ser Ile Glu Glu Thr Asp Gly Tyr Ser Ala Ser Trp Thr
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Val Pro Phe Gly Ala Arg Ala Tyr Val Glu Met Met Gln Cys Gln Ala
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Glu Lys Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro
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Leu His Gly Cys Ala Val Asp Lys Leu Gly Arg Cys Lys Arg Asp Asp
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Claims

1. A consensus protein which has the amino acid sequence shown in Figure 2 or any variants thereof wherein the variants basic properties such as enzymatic activity (type of and specific activity), thermostability, activity in a certain pH-range (pH-stability) have not significantly been changed.

2. The consensus protein of claim 1 characterized therein that in the amino acid sequence of Figure 2 the following replacements have been effected Q50L, Q50T, Q50G, Q50T-Y51N or Q50L-Y51N.

3. A food, feed or pharmaceutical composition comprising a consensus protein as claimed in claims 1 or 2.

Patentansprüche

1. Konsensusprotein, das die in Figur 2 gezeigte Aminosäuresequenz hat, oder Varianten davon, wobei die Grundeigenschaften der Varianten wie enzymatische Aktivität (Art und spezifische Aktivität), Thermostabilität, Aktivität in einem bestimmten pH-Bereich (pH-Stabilität) nicht signifikant verändert worden sind.

2. Konsensusprotein nach Anspruch 1, **dadurch gekennzeichnet, dass** in der Aminosäuresequenz von Figur 2 die folgenden Austausche durchgeführt worden sind: Q50L, Q50T, Q50G, Q50T-Y51N oder Q50L-Y51N.

3. Nahrungsmittel, Futtermittel oder Arzneimittel umfassend ein Konsensusprotein nach Anspruch 1 oder 2.

Revendications

1. Protéine consensus qui comporte la séquence d'acides aminés représentée à la Fig. 2 ou l'une quelconque des variantes de celle-ci, dans laquelle les propriétés de base des variantes telles qu'activité enzymatique (type et activité spécifique), thermostabilité, activité dans une certaine plage de pH (stabilité au pH) n'ont pas été changées de façon significative.

2. Protéine consensus suivant la revendication 1, **caractérisée en ce que** dans la séquence d'acides aminés de la Fig. 2 les remplacements suivants ont été effectués Q50L, Q50T, Q50G, Q50T-Y51N ou Q50L-Y51N.

3. Aliment, alimentation ou composition pharmaceutique comprenant une protéine consensus suivant l'une ou l'autre des revendications 1 et 2.

Figure 1/1

	1				50
A. terreus 9A-1	KhsDCNSVDh	GYQCFPELSH	kWGlYAPYFS	LQDESPFPlD	VPEDChITFV
A. terreus cbs	NhsDCTSVDr	GYQCFPELSH	kWGlYAPYFS	LQDESPFPlD	VPDDChITFV
A. niger var. *awamori*	NqsTCDTVDQ	GYQCFSETSH	LWGQYAPFFS	LANESAISPD	VPAGCrVTFA
A. niger T213	NqsSCDTVDQ	GYQCFSETSH	LWGQYAPFFS	LANESVISPD	VPAGCrVTFA
A. niger NRRL3135	NqsSCDTVDQ	GYQCFSETSH	LWGQYAPFFS	LANESVISPE	VPAGCrVTFA
A. fumigatus 13073	GSkSCDTVDl	GYQCsPATSH	LWGQYSPFFS	LEDElSVSSK	LPKDCrITLV
A. fumigatus 32722	GSkSCDTVDl	GYQCsPATSH	LWGQYSPFFS	LEDElSVSSK	LPKDCrITLV
A. fumigatus 58128	GSkSCDTVDl	GYQCsPATSH	LWGQYSPFFS	LEDElSVSSK	LPKDCrITLV
A. fumigatus 26906	GSkSCDTVDl	GYQCsPATSH	LWGQYSPFFS	LEDElSVSSK	LPKDCrITLV
A. fumigatus 32239	GSkACDTVEl	GYQCsPGTSH	LWGQYSPFFS	LEDElSVSSD	LPKDCrVTFV
A. nidulans	QNHSCNTADG	GYQCFPNVSH	VWGQYSPYFS	IEQESAISeD	VPHGCeVTFV
T. thermophilus	DSHSCNTVEG	GYQCrPEISH	sWGQYSPFFS	LADQSEISPD	VPQNCkITFV
M. thermophila	ESRPCDTpDl	GFQCgTAISH	FWGQYSPYFS	VpSElDaS..	IPDDCeVTFA
Consensus	NSHSCDTVDG	GYQCFPEISH	LWGQYSPYFS	LEDESAISPD	VPDDC-VTFV
Consensus phytase	**NSHSCDTVDG**	**GYQCFPEISH**	**LWGQYSPYFS**	**LEDESAISPD**	**VPDDCRVTFV**

	51				100
A. terreus 9A-1	QVLARHGARs	PThSKtKAYA	AtIAAIQKSA	TaFpGKYAFL	QSYNYSLDSE
A. terreus cbs	QVLARHGARs	PTDSKtKAYA	AtIAAIQKNA	TaLpGKYAFL	KSYNYSMGSE
A. niger var. *awamori*	QVLSRHGARY	PTESKgKkYS	ALIEEIQQNV	TtFDGKYAFL	KTYNYSLGAD
A. niger T213	QVLSRHGARY	PTESKgKkYS	ALIEEIQQNV	TtFDGKYAFL	KTYNYSLGAD
A. niger NRRL3135	QVLSRHGARY	PTDSKgKkYS	ALIEEIQQNA	TtFDGKYAFL	KTYNYSLGAD
A. fumigatus 13073	QVLSRHGARY	PTSSKsKkYK	kLVTAIQaNA	TdFKGKFAFL	KTYNYTLGAD
A. fumigatus 32722	QVLSRHGARY	PTSSKsKkYK	kLVTAIQaNA	TdFKGKFAFL	KTYNYTLGAD
A. fumigatus 58128	QVLSRHGARY	PTSSKsKkYK	kLVTAIQaNA	TdFKGKFAFL	KTYNYTLGAD
A. fumigatus 26906	QVLSRHGARY	PTSSKsKkYK	kLVTAIQaNA	TdFKGKFAFL	KTYNYTLGAD
A. fumigatus 32239	QVLSRHGARY	PTASKsKkYK	kLVTAIQKNA	TeFKGKFAFL	ETYNYTLGAD
A. nidulans	QVLSRHGARY	PTESKsKAYS	GLIEAIQKNA	TsFwGQYAFL	ESYNYTLGAD
T. thermophilus	QLLSRHGARY	PTSSKtElYS	QLISrIQKTA	TaYKGyYAFL	KDYrYqLGAN
M. thermophila	QVLSRHGARa	PTlKRaaSYv	DLIDrIHhGA	IsYgPgYEFL	RTYDYTLGAD
Consensus	QVLSRHGARY	PTSSK-KAYS	ALIEAIQKNA	T-FKGKYAFL	KTYNYTLGAD
Consensus phytase	**QVLSRHGARY**	**PTSSKSKAYS**	**ALIEAIQKNA**	**TAFKGKYAFL**	**KTYNYTLGAD**

	101				150
A. terreus 9A-1	ELTPFGrNQL	rDlGaQFYeR	YNALTRhInP	FVRATDASRV	hESAEKFVEG
A. terreus cbs	NLTPFGrNQL	qDlGaQFYRR	YDTLTRhInP	FVRAADSSRV	hESAEKFVEG
A. niger var. *awamori*	DLTPFGEQEL	VNSGIKFYQR	YESLTRNIIP	FIRSSGSSRV	IASGEKFIEG
A. niger T213	DLTPFGEQEL	VNSGIKFYQR	YESLTRNIIP	FIRSSGSSRV	IASGEKFIEG
A. niger NRRL3135	DLTPFGEQEL	VNSGIKFYQR	YESLTRNIVP	FIRSSGSSRV	IASGKKFIEG
A. fumigatus 13073	DLTPFGEQQL	VNSGIKFYQR	YKALARSVVP	FIRASGSDRV	IASGEKFIEG
A. fumigatus 32722	DLTPFGEQQL	VNSGIKFYQR	YKALARSVVP	FIRASGSDRV	IASGEKFIEG
A. fumigatus 58128	DLTPFGEQQL	VNSGIKFYQR	YKALARSVVP	FIRASGSDRV	IASGEKFIEG
A. fumigatus 26906	DLTAFGEQQL	VNSGIKFYQR	YKALARSVVP	FIRASGSDRV	IASGEKFIEG
A. fumigatus 32239	DLTPFGEQQM	VNSGIKFYQK	YKALAgSVVP	FIRSSGSDRV	IASGEKFIEG
A. nidulans	DLTiFGENQM	VDSGaKFYRR	YKNLARKnTP	FIRASGSDRV	VASAEKFING
T. thermophilus	DLTPFGENQM	IQlGIKFYnH	YKSLARNaVP	FVRCSGSDRV	IASGrlFIEG
M. thermophila	ELTRtGQQQM	VNSGIKFYRR	YRALARKsIP	FVRTAGqDRV	VhSAENFTQG
Consensus	DLTPFGENQM	VNSGIKFYRR	YKALARK-VP	FVRASGSDRV	IASAEKFIEG
Consensus phytase	**DLTPFGENQM**	**VNSGIKFYRR**	**YKALARKIVP**	**FIRASGSDRV**	**IASAEKFIEG**

Figure 1/2

```
                          151                                                     200
A. terreus 9A-1           FQTARqDDHh ANpHQPSPrV DVaIPEGSAY NNTLEHSlCT AFES...STV
A. terreus cbs            FQNARqGDPh ANpHQPSPrV DVVIPEGTAY NNTLEHSICT AFEA...STV
A. niger var. awamori     FQSTKLkDPr AqpgQSSPkI DVVISEASSs NNTLDPGTCT VFED...SEL
A. niger T213             FQSTKLkDPr AqpgQSSPkI DVVISEASSs NNTLDPGTCT VFED...SEL
A. niger NRRL3135         FQSTKLkDPr AqpgQSSPkI DVVISEASSs NNTLDPGTCT VFED...SEL
A. fumigatus 13073        FQqAKLADPG A.TNRAAPAI SVIIPESETF NNTLDHGVCT kFEA...SQL
A. fumigatus 32722        FQqAKLADPG A.TNRAAPAI SVIIPESETF NNTLDHGVCT kFEA...SQL
A. fumigatus 58128        FQqAKLADPG A.TNRAAPAI SVIIPESETF NNTLDHGVCT kFEA...SQL
A. fumigatus 26906        FQqAKLADPG A.TNRAAPAI SVIIPESETF NNTLDHGVCT kFEA...SQL
A. fumigatus 32239        FQqANVADPG A.TNRAAPVI SVIIPESETY NNTLDHSVCT NFEA...SEL
A. nidulans               FRKAQLhDHG S..gQATPVV NVIIPEiDGF NNTLDHSTCV SFEN...DEr
T. thermophilus           FQSAKVlDPh SDkHDAPPTI NVIIeEGPSY NNTLDtGSCP VFED...SSg
M. thermophila            FHSAlLADRG STvRPTlPyd mVVIPETAGa NNTLHNDlCT AFEEgpySTI

Consensus                 FQSAKLADPG S-PHQASPVI NVIIPEGSGY NNTLDHGTCT AFED---SEL
Consensus phytase         FQSAKLADPG SQPHQASPVI DVIIPEGSGY NNTLDHGTCT AFED...SEL


                          201                                                     250
A. terreus 9A-1           GDDAvANFTA VFAPAIaQRL EADLPGVqLS TDDVVnLMAM CPFETVSlTD
A. terreus cbs            GDAAADNFTA VFAPAIakRL EADLPGVqLS ADDVVnLMAM CPFETVSlTD
A. niger var. awamori     ADTVEANFTA TFAPSIRQRL ENDLSGVTLT DTEVTyLMDM CSFDTIStST
A. niger T213             ADTVEANFTA TFAPSIRQRL ENDLSGVTLT DTEVTyLMDM CSFDTIStST
A. niger NRRL3135         ADTVEANFTA TFVPSIRQRL ENDLSGVTLT DTEVTyLMDM CSFDTIStST
A. fumigatus 13073        GDEVAANFTA lFAPDIRARa EkHLPGVTLT DEDVVsLMDM CSFDTVARTS
A. fumigatus 32722        GDEVAANFTA lFAPDIRARa EkHLPGVTLT DEDVVsLMDM CSFDTVARTS
A. fumigatus 58128        GDEVAANFTA lFAPDIRARa EkHLPGVTLT DEDVVsLMDM CSFDTVARTS
A. fumigatus 26906        GDEVAANFTA lFAPDIRARa KkHLPGVTLT DEDVVsLMDM CSFDTVARTS
A. fumigatus 32239        GDEVEANFTA lFAPAIRARI EkHLPGVqLT DDDVVsLMDM CSFDTVARTA
A. nidulans               ADEiEANFTA IMGPPIRkRL ENDLPGIKLT NENVIyLMDM CSFDTMARTA
T. thermophilus           GHDAQEKFAk qFAPAIlEKI KDHLPGVDLA vSDVpyLMDL CPFETLARNh
M. thermophila            GDDAQDTYlS TFAGPItARV NANLPGANLT DADTVaLMDL CPFETVAsSS

Consensus                 GDDAEANFTA TFAPAIRARL EADLPGVTLT DEDVV-LMDM CPFETVARTS
Consensus phytase         GDDVEANFTA LFAPAIRARL EADLPGVTLT DEDVVYLMDM CPFETVARTS


                          251                                                     300
A. terreus 9A-1           .......... ...DAhTLSPFC DLFTAtEWtq YNYLlSLDKY YGYGGGNPLG
A. terreus cbs            .......... ...DAhTLSPFC DLFTAaEWtq YNYLlSLDKY YGYGGGNPLG
A. niger var. awamori     .......... ...vDTKLSPFC DLFTHdEWih YDYLQSLkKY YGHGAGNPLG
A. niger T213             .......... ...vDTKLSPFC DLFTHdEWih YDYLRSLkKY YGHGAGNPLG
A. niger NRRL3135         .......... ...vDTKLSPFC DLFTHdEWin YDYLQSLkKY YGHGAGNPLG
A. fumigatus 13073        .......... ...DASQLSPFC QLFTHnEWkk YNYLQSLGKY YGYGAGNPLG
A. fumigatus 32722        .......... ...DASQLSPFC QLFTHnEWkk YNYLQSLGKY YGYGAGNPLG
A. fumigatus 58128        .......... ...DASQLSPFC QLFTHnEWkk YNYLQSLGKY YGYGAGNPLG
A. fumigatus 26906        .......... ...DASQLSPFC QLFTHnEWkk YNYLQSLGKY YGYGAGNPLG
A. fumigatus 32239        .......... ...DASELSPFC AIFTHnEWkk YDYLQSLGKY YGYGAGNPLG
A. nidulans               .......... ...HGTELSPFC AIFTEkEWlq YDYLQSLSKY YGYGAGSPLG
T. thermophilus           .......... ...TDT.LSPFC ALsTQeEWqa YDYYQSLGKY YGnGGGNPLG
M. thermophila            sdpatadagg gNGrpLSPFC rLFSEsEWra YDYLQSVGKW YGYGPGNPLG

Consensus                 ---------- -DATELSPFC ALFTE-EW-- YDYLQSLGKY YGYGAGNPLG
Consensus phytase         .......... .DATELSPFC ALFTHDEWRQ YDYLQSLGKY YGYGAGNPLG
```

Figure 1/3

```
                          301                                                   350
A. terreus 9A-1           PVQGVGWaNE LMARLTRAPV HDHTCVNNTL DASPATFPLN ATLYADFSHD
A. terreus cbs            PVQGVGWaNE LIARLTRSPV HDHTCVNNTL DANPATFPLN ATLYADFSHD
A. niger var. awamori     PTQGVGYaNE LIARLTHSPV HDDTSSNHTL DSNPATFPLN STLYADFSHD
A. niger T213             PTQGVGYaNE LIARLTHSPV HDDTSSNHTL DSNPATFPLN STLYADFSHD
A. niger NRRL3135         PTQGVGYaNE LIARLTHSPV HDDTSSNHTL DSSPATFPLN STLYADFSHD
A. fumigatus 13073        PAQGIGFtNE LIARLTRSPV QDHTSTNsTL vSNPATFPLN ATMYVDFSHD
A. fumigatus 32722        PAQGIGFtNE LIARLTRSPV QDHTSTNsTL vSNPATFPLN ATMYVDFSHD
A. fumigatus 58128        PAQGIGFtNE LIARLTRSPV QDHTSTNsTL vSNPATFPLN ATMYVDFSHD
A. fumigatus 26906        PAQGIGFtNE LIARLTRSPV QDHTSTNsTL vSNPATFPLN ATMYVDFSHD
A. fumigatus 32239        PAQGIGFtNE LIARLTNSPV QDHTSTNsTL DSDPATFPLN ATIYVDFSHD
A. nidulans               PAQGIGFtNE LIARLTQSPV QDNTSTNHTL DSNPATFPLD rKLYADFSHD
T. thermophilus           PAQGVGFvNE LIARMTHSPV QDYTTVNHTL DSNPATFPLN ATLYADFSHD
M. thermophila            PTQGVGFvNE LLARLAgvPV RDgTSTNRTL DGDPrTFPLG rPLYADFSHD

Consensus                 PAQGVGF-NE LIARLTHSPV QDHTSTNHTL DSNPATFPLN ATLYADFSHD
Consensus phytase         PAQGVGFANE LIARLTRSPV QDHTSTNHTL DSNPATFPLN ATLYADFSHD

                          351                                                   400
A. terreus 9A-1           SNLVSIFWAL GLYNGTAPLS qTSVESVSQT DGYAAAWTVP FAARAYVEMM
A. terreus cbs            SNLVSIFWAL GLYNGTkPLS qTTVEDITrT DGYAAAWTVP FAARAYIEMM
A. niger var. awamori     NGIISILFAL GLYNGTkPLS TTTVENITQT DGFSSAWTVP FASRlYVEMM
A. niger T213             NGIISILFAL GLYNGTkPLS TTTVENITQT DGFSSAWTVP FASRlYVEMM
A. niger NRRL3135         NGIISILFAL GLYNGTkPLS TTTVENITQT DGFSSAWTVP FASRlYVEMM
A. fumigatus 13073        NSMVSIFFAL GLYNGTEPLS rTSVESaKEl DGYSASWVVP FGARAYFEtM
A. fumigatus 32722        NSMVSIFFAL GLYNGTGPLS rTSVESaKEl DGYSASWVVP FGARAYFEtM
A. fumigatus 58128        NSMVSIFFAL GLYNGTEPLS rTSVESaKEl DGYSASWVVP FGARAYFEtM
A. fumigatus 26906        NSMVSIFFAL GLYNGTEPLS rTSVESaKEl DGYSASWVVP FGARAYFEtM
A. fumigatus 32239        NGMIPIFFAM GLYNGTEPLS qTSeESTKES NGYSASWAVP FGARAYFEtM
A. nidulans               NSMISIFFAM GLYNGTQPLS mDSVESIQEm DGYAASWTVP FGARAYFELM
T. thermophilus           NTMTSIFaAL GLYNGTAkLS TTEIKSIEET DGYSAAWTVP FGGRAYIEMM
M. thermophila            NDMMGVLgAL GaYDGVPPLD KTArrDpEEl GGYAASWAVP FAARiYVEKM

Consensus                 NSMISIFFAL GLYNGTAPLS TTSVESIEET DGYAASWTVP FGARAYVEMM
Consensus phytase         NSMISIFFAL GLYNGTAPLS TTSVESIEET DGYSASWTVP FGARAYVEMM

                          401                                                   450
A. terreus 9A-1           QC........ .....RAEKE PLVRVLVNDR VMPLHGCPTD KLGRCKrDAF
A. terreus cbs            QC........ .....RAEKQ PLVRVLVNDR VMPLHGCAVD NLGRCKrDDF
A. niger var. awamori     QC........ .....QAEQE PLVRVLVNDR VVPLHGCPID aLGRCTrDSF
A. niger T213             QC........ .....QAEQE PLVRVLVNDR VVPLHGCPID aLGRCTrDSF
A. niger NRRL3135         QC........ .....QAEQE PLVRVLVNDR VVPLHGCPVD aLGRCTrDSF
A. fumigatus 13073        QC........ .....KSEKE PLVRALINDR VVPLHGCDVD KLGRCKLNDF
A. fumigatus 32722        QC........ .....KSEKE PLVRALINDR VVPLHGCDVD KLGRCKLNDF
A. fumigatus 58128        QC........ .....KSEKE SLVRALINDR VVPLHGCDVD KLGRCKLNDF
A. fumigatus 26906        QC........ .....KSEKE PLVRALINDR VVPLHGCDVD KLGRCKLNDF
A. fumigatus 32239        QC........ .....KSEKE PLVRALINDR VVPLHGCAVD KLGRCKLKDF
A. nidulans               QC........ .....E.KKE PLVRVLVNDR VVPLHGCAVD KFGRCTLDDW
T. thermophilus           QC........ .....DDSDE PVVRVLVNDR VVPLHGCEVD SLGRCKrDDF
M. thermophila            RCsggggggg ggegrQEKDE eMVRVLVNDR VMTLkGCGAD ErGMCTLErF

Consensus                 QC-------- -----QAEKE PLVRVLVNDR VVPLHGCAVD KLGRCKLDDF
Consensus phytase         QC........ .....QAEKE PLVRVLVNDR VVPLHGCAVD KLGRCKRDDF
```

Figure 1/4

	451 471
A. terreus 9A-1	VAGLSFAQAG GNWADCF~~~ ~
A. terreus cbs	VEGLSFARAG GNWAECF~~~ ~
A. niger var. *awamori*	VrGLSFARSG GDWAECsA~~ ~
A. niger T213	VrGLSFARSG GDWAECFA~~ ~
A. niger NRRL3135	VrGLSFARSG GDWAECFA~~ ~
A. fumigatus 13073	VKGLSWARSG GNWGECFS~~ ~
A. fumigatus 32722	VKGLSWARSG GNWGECFS~~ ~
A. fumigatus 58128	VKGLSWARSG GNWGECFS~~ ~
A. fumigatus 26906	VKGLSWARSG GNWGECFS~~ ~
A. fumigatus 32239	VKGLSWARSG GNSEQSFS~~ ~
A. nidulans	VEGLNFARSG GNWkTCFTl~ ~
T. thermophilus	VrGLSFARqG GNWEGCYAas e
M. thermophila	IESMAFARGN GKWDlCFA~~ ~
Consensus	VEGLSFARSG GNWAECFA-- -
Consensus phytase	VEGLSFARSG GNWAECFA.. .

Figure 2/1

```
    CP-1
      Eco RI  M   G   V   F   V   V   L   L   S   I   A   T   L   F   G   S   T
    TATATGAATTCATGGGCGTGTTCGTCGTGCTACTGTCCATTGCCACCTTGTTCGGTTCCA
  1 ---------+---------+---------+---------+---------+---------+ 60
    ATATACTTAAGTACCCGCACAAGCAGCACGATGACAGGTAACGGTGGAACAAGCCAAGGT

      S  G  T  A  L  G  P  R  G  N  S  H  S  C  D  T  V  D  G  G
    CATCCGGTACCGCCTTGGGTCCTCGTGGTAATTCTCACTCTTGTGACACTGTTGACGGTG
 61 ---------+---------+---------+---------+---------+---------+ 120
    GTAGGCCATGGCGGAACCCAGGAGCACCATTAAGAGTGAGAACACTGTGACAACTGCCAC
        CP-2
            CP-3
      Y  Q  C  F  P  E  I  S  H  L  W  G  Q  Y  S  P  Y  F  S  L
    GTTACCAATGTTTCCCAGAAATTTCTCACTTGTGGGGTCAATACTCTCCATACTTCTCTT
121 ---------+---------+---------+---------+---------+---------+ 180
    CAATGGTTACAAAGGGTCTTTAAAGAGTGAACACCCCAGTTATGAGAGGTATGAAGAGAA

      E  D  E  S  A  I  S  P  D  V  P  D  D  C  R  V  T  F  V  Q
    TGGAAGACGAATCTGCTATTTCTCCAGACGTTCCAGACGACTGTAGAGTTACTTTCGTTC
181 ---------+---------+---------+---------+---------+---------+ 240
    ACCTTCTGCTTAGACGATAAAGAGGTCTGCAAGGTCTGCTGACATCTCAATGAAAGCAAG
              CP-4
                  CP-5
      V  L  S  R  H  G  A  R  Y  P  T  S  S  K  S  K  A  Y  S  A
    AAGTTTTGTCTAGACACGGTGCTAGATACCCAACTTCTTCTAAGTCTAAGGCTTACTCTG
241 ---------+---------+---------+---------+---------+---------+ 300
    TTCAAAACAGATCTGTGCCACGATCTATGGGTTGAAGAAGATTCAGATTCCGAATGAGAC

      L  I  E  A  I  Q  K  N  A  T  A  F  K  G  K  Y  A  F  L  K
    CTTTGATTGAAGCTATTCAAAAGAACGCTACTGCTTTCAAGGGTAAGTACGCTTTCTTGA
301 ---------+---------+---------+---------+---------+---------+ 360
    GAAACTAACTTCGATAAGTTTTCTTGCGATGACGAAAGTTCCCATTCATGCGAAAGAACT
                          CP-6
                              CP-7
      T  Y  N  Y  T  L  G  A  D  D  L  T  P  F  G  E  N  Q  M  V
    AGACTTACAACTACACTTTGGGTGCTGACGACTTGACTCCATTCGGTGAAAACCAAATGG
361 ---------+---------+---------+---------+---------+---------+ 420
    TCTGAATGTTGATGTGAAACCCACGACTGCTGAACTGAGGTAAGCCACTTTTGGTTTACC

      N  S  G  I  K  F  Y  R  R  Y  K  A  L  A  R  K  I  V  P  F
    TTAACTCTGGTATTAAGTTCTACAGAAGATACAAGGCTTTGGCTAGAAAGATTGTTCCAT
421 ---------+---------+---------+---------+---------+---------+ 480
    AATTGAGACCATAATTCAAGATGTCTTCTATGTTCCGAAACCGATCTTTCTAACAAGGTA
                                    CP-8
                                        CP-9
      I  R  A  S  G  S  D  R  V  I  A  S  A  E  K  F  I  E  G  F
    TCATTAGAGCTTCTGGTTCTGACAGAGTTATTGCTTCTGCTGAAAAGTTCATTGAAGGTT
481 ---------+---------+---------+---------+---------+---------+ 540
    AGTAATCTCGAAGACCAAGACTGTCTCAATAACGAAGACGACTTTTCAAGTAACTTCCAA

      Q  S  A  K  L  A  D  P  G  S  Q  P  H  Q  A  S  P  V  I  D
    TCCAATCTGCTAAGTTGGCTGACCCAGGTTCTCAACCACACCAAGCTTCTCCAGTTATTG
541 ---------+---------+---------+---------+---------+---------+ 600
    AGGTTAGACGATTCAACCGACTGGGTCCAAGAGTTGGTGTGGTTCGAAGAGGTCAATAAC
                                                  CP-10
                                                      CP-11
      V  I  I  P  E  G  S  G  Y  N  N  T  L  D  H  G  T  C  T  A
    ACGTTATTATTCCAGAAGGaTCcGGTTACAACAACACTTTGGACCACGGTACTTGTACTG
601 ---------+---------+---------+---------+---------+---------+ 660
    TGCAATAATAAGGTCTTCCtAGgCCAATGTTGTTGTGAAACCTGGTGCCATGAACATGAC
```

Figure 2/2

```
      F  E  D  S  E  L  G  D  D  V  E  A  N  F  T  A  L  F  A  P
     CTTTCGAAGACTCTGAATTGGGTGACGACGTTGAAGCTAACTTCACTGCTTTGTTCGCTC
 661 ---------+---------+---------+---------+---------+---------+ 720
     GAAAGCTTCTGAGACTTAACCCACTGCTGCAACTTCGATTGAAGTGACGAAACAAGCGAG
                                                            CP-12

      A  I  R  A  R  L  E  A  D  L  P  G  V  T  L  T  D  E  D  V
     CAGCTATTAGAGCTAGATTGGAAGCTGACTTGCCAGGTGTTACTTTGACTGACGAAGACG
 721 ---------+---------+---------+---------+---------+---------+ 780
     GTCGATAATCTCGATCTAACCTTCGACTGAACGGTCCACAATGAAACTGACTGCTTCTGC

     CP-13
      V  Y  L  M  D  M  C  P  F  E  T  V  A  R  T  S  D  A  T  E
     TTGTTTACTTGATGGACATGTGTCCATTCGAAACTGTTGCTAGAACTTCTGACGCTACTG
 781 ---------+---------+---------+---------+---------+---------+ 840
     AACAAATGAACTACCTGTACACAGGTAAGCTTTGACAACGATCTTGAAGACTGCGATGAC

      L  S  P  F  C  A  L  F  T  H  D  E  W  R  Q  Y  D  Y  L  Q
     AATTGTCTCCATTCTGTGCTTTGTTCACTCACGACGAATGGAGACAATACGACTACTTGC
 841 ---------+---------+---------+---------+---------+---------+ 900
     TTAACAGAGGTAAGACACGAAACAAGTGAGTGCTGCTTACCTCTGTTATGCTGATGAACG
         CP-14
              CP-15
      S  L  G  K  Y  Y  G  Y  G  A  G  N  P  L  G  P  A  Q  G  V
     AATCTTTGGGTAAGTACTACGGTTACGGTGCTGGTAACCCATTGGGTCCAGCTCAAGGTG
 901 ---------+---------+---------+---------+---------+---------+ 960
     TTAGAAACCCATTCATGATGCCAATGCCACGACCATTGGGTAACCCAGGTCGAGTTCCAC

      G  F  A  N  E  L  I  A  R  L  T  R  S  P  V  Q  D  H  T  S
     TTGGTTTCGCTAACGAATTGATTGCTAGATTGACTAGATCTCCAGTTCAAGACCACACTT
 961 ---------+---------+---------+---------+---------+---------+ 1020
     AACCAAAGCGATTGCTTAACTAACGATCTAACTGATCTAGAGGTCAAGTTCTGGTGTGAA
                  CP-16
                       CP-17
      T  N  H  T  L  D  S  N  P  A  T  F  P  L  N  A  T  L  Y  A
     CTACTAACCACACTTTGGACTCTAACCCAGCTACTTTCCCATTGAACGCTACTTTGTACG
1021 ---------+---------+---------+---------+---------+---------+ 1080
     GATGATTGGTGTGAAACCTGAGATTGGGTCGATGAAAGGGTAACTTGCGATGAAACATGC

      D  F  S  H  D  N  S  M  I  S  I  F  F  A  L  G  L  Y  N  G
     CTGACTTCTCTCACGACAACTCTATGATTTCTATTTTCTTCGCTTTGGGTTTGTACAACG
1081 ---------+---------+---------+---------+---------+---------+ 1140
     GACTGAAGAGAGTGCTGTTGAGATACTAAAGATAAAAGAAGCGAAACCCAAACATGTTGC
                             CP-18
                                  CP-19
      T  A  P  L  S  T  T  S  V  E  S  I  E  E  T  D  G  Y  S  A
     GTACTGCTCCATTGTCTACTACTTCTGTTGAATCTATTGAAGAAACTGACGGTTACTCTG
1141 ---------+---------+---------+---------+---------+---------+ 1200
     CATGACGAGGTAACAGATGATGAAGACAACTTAGATAACTTCTTTGACTGCCAATGAGAC

      S  W  T  V  P  F  G  A  R  A  Y  V  E  M  M  Q  C  Q  A  E
     CTTCTTGGACTGTTCCATTCGGTGCTAGAGCTTACGTTGAAATGATGCAATGTCAAGCTG
1201 ---------+---------+---------+---------+---------+---------+ 1260
     GAAGAACCTGACAAGGTAAGCCACGATCTCGAATGCAACTTTACTACGTTACAGTTCGAC
                                                 CP-20
                                                      CP-21
      K  E  P  L  V  R  V  L  V  N  D  R  V  V  P  L  H  G  C  A
     AAAAGGAACCATTGGTTAGAGTTTTGGTTAACGACAGAGTTGTTCCATTGCACGGTTGTG
1261 ---------+---------+---------+---------+---------+---------+ 1320
     TTTTCCTTGGTAACCAATCTCAAAACCAATTGCTGTCTCAACAAGGTAACGTGCCAACAC
```

Figure 2/3

```
        V  D  K  L  G  R  C  K  R  D  D  F  V  E  G  L  S  F  A  R
      CTGTTGACAAGTTGGGTAGATGTAAGAGAGACGACTTCGTTGAAGGTTTGTCTTTCGCTA
1321  ---------+---------+---------+---------+---------+---------+  1380
      GACAACTGTTCAACCCATCTACATTCTCTCTGCTGAAGCAACTTCCAAACAGAAAGCGAT
                                                          CP-22

        S  G  G  N  W  A  E  C  F  A  *  Eco RI
      GATCTGGTGGTAACTGGGCTGAATGTTTCGCTTAAGAATTCATATA
1381  ---------+---------+---------+---------+------  1426
      CTAGACCACCATTGACCCGACTTACAAAGCGAATTCTTAAGTATAT
```

Figure 3

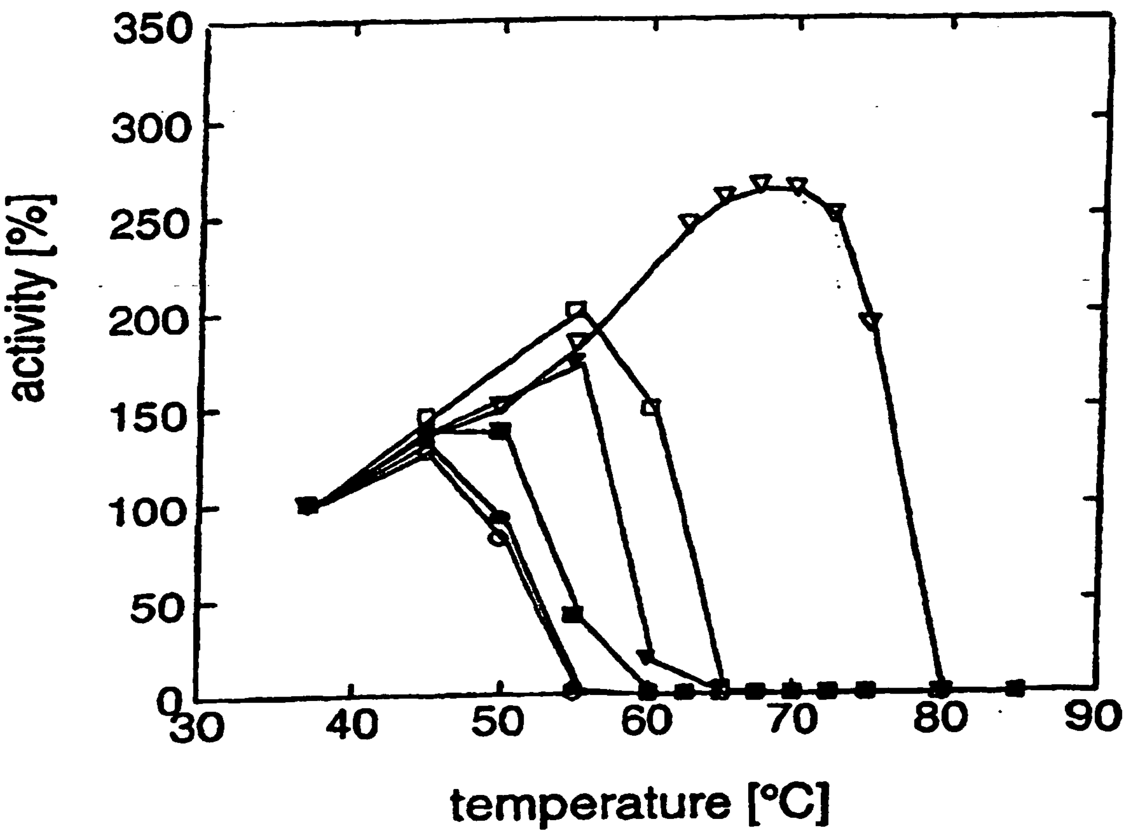

350
300
250
200
150
100
50
0
activity [%]
30
40
50
60
70
80
90
temperature [°C]

Figure 4

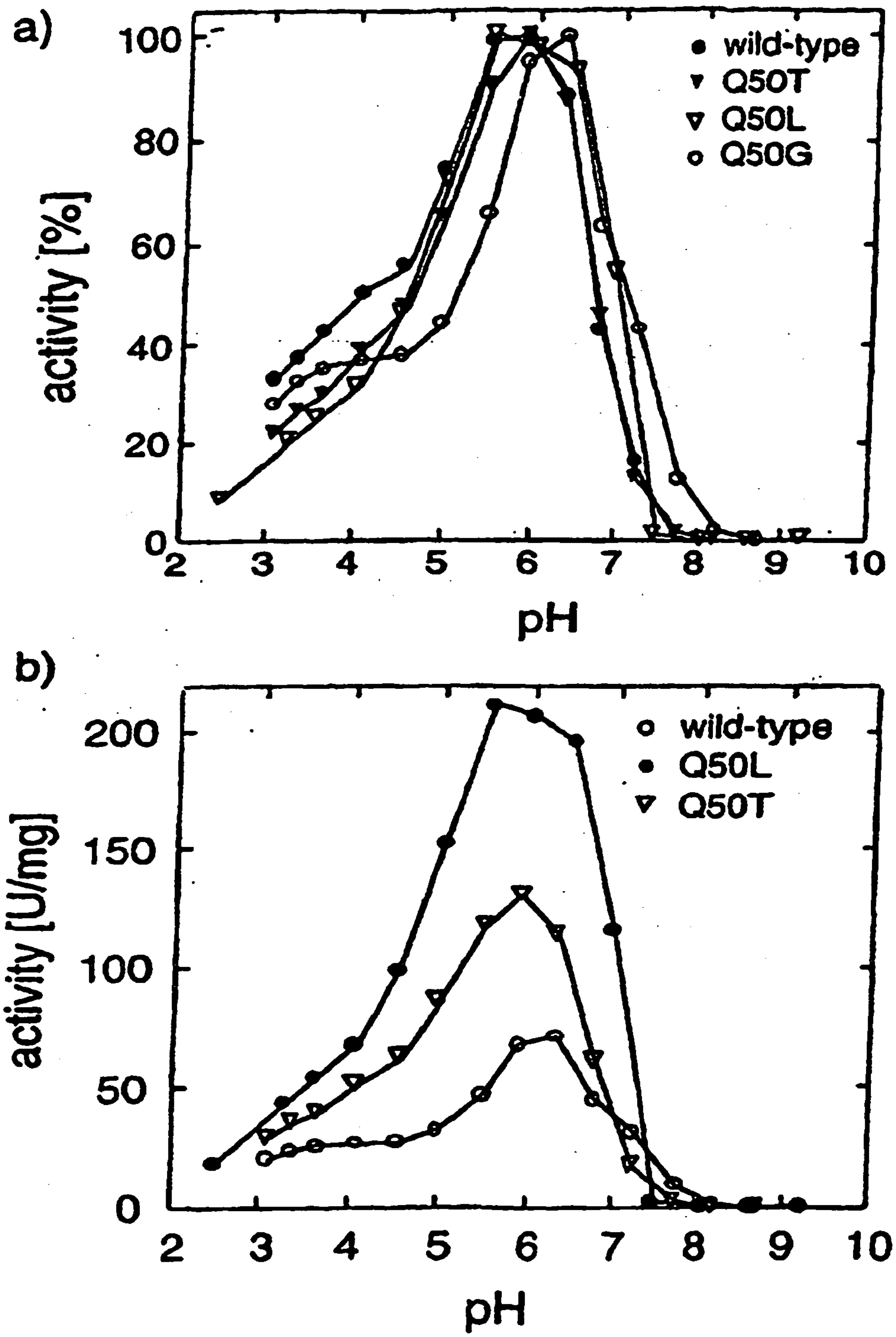
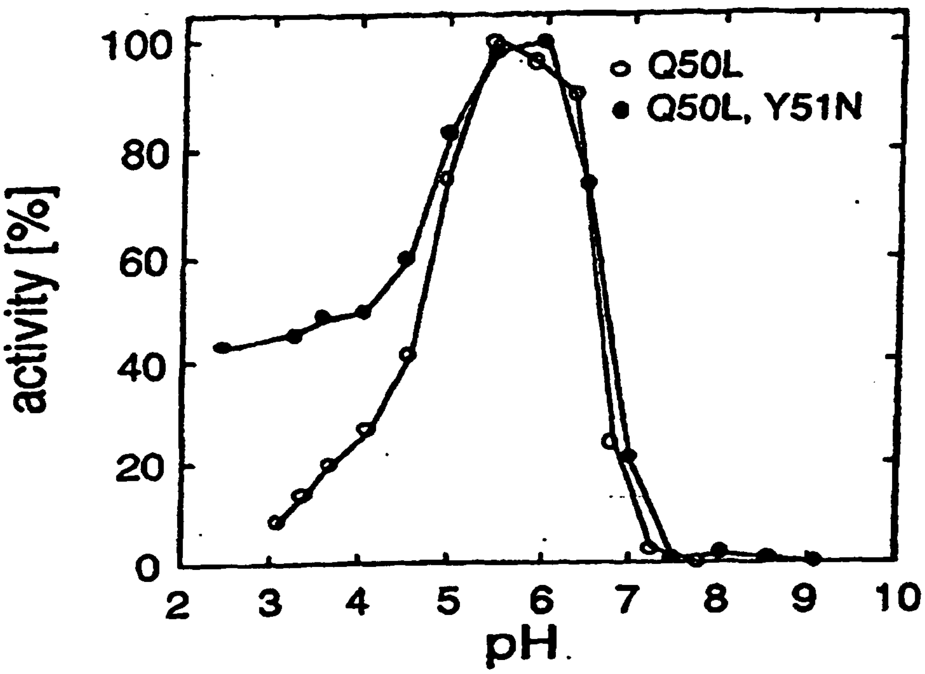

a)
wild-type
Q50T
Q50L
Q50G
activity [%]
pH
b)
wild-type
Q50L
Q50T
activity [U/mg]
pH

Figure 5

a)


100
80
60
40
20
0
2
3
4
5
6
7
8
9
10
pH
activity [%]
Q50L
Q50L, Y51N

b)

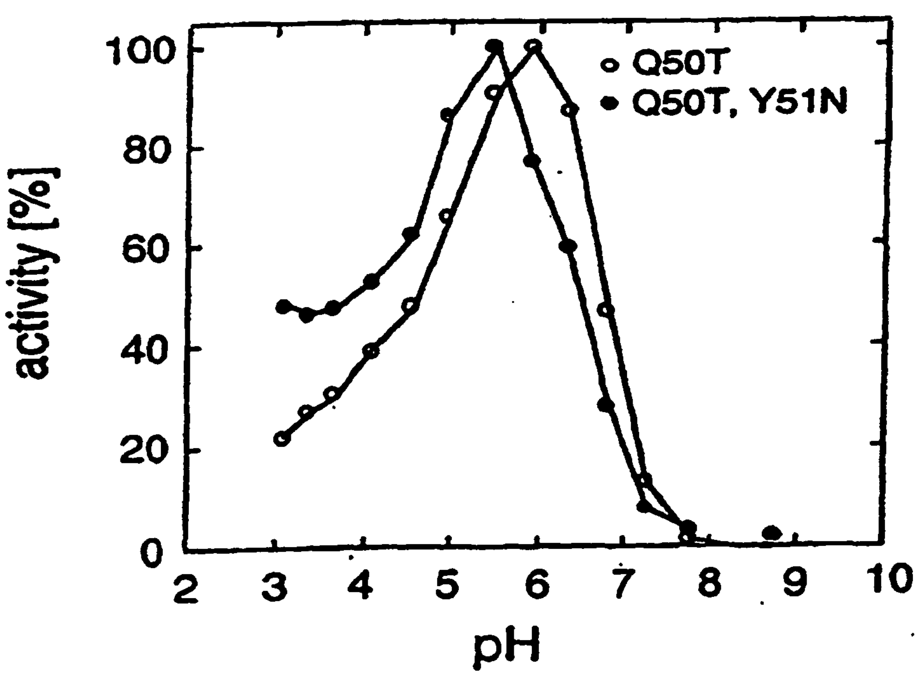

100
80
60
40
20
0
2
3
4
5
6
7
8
9
10
pH
activity [%]
Q50T
Q50T, Y51N

Figure 6

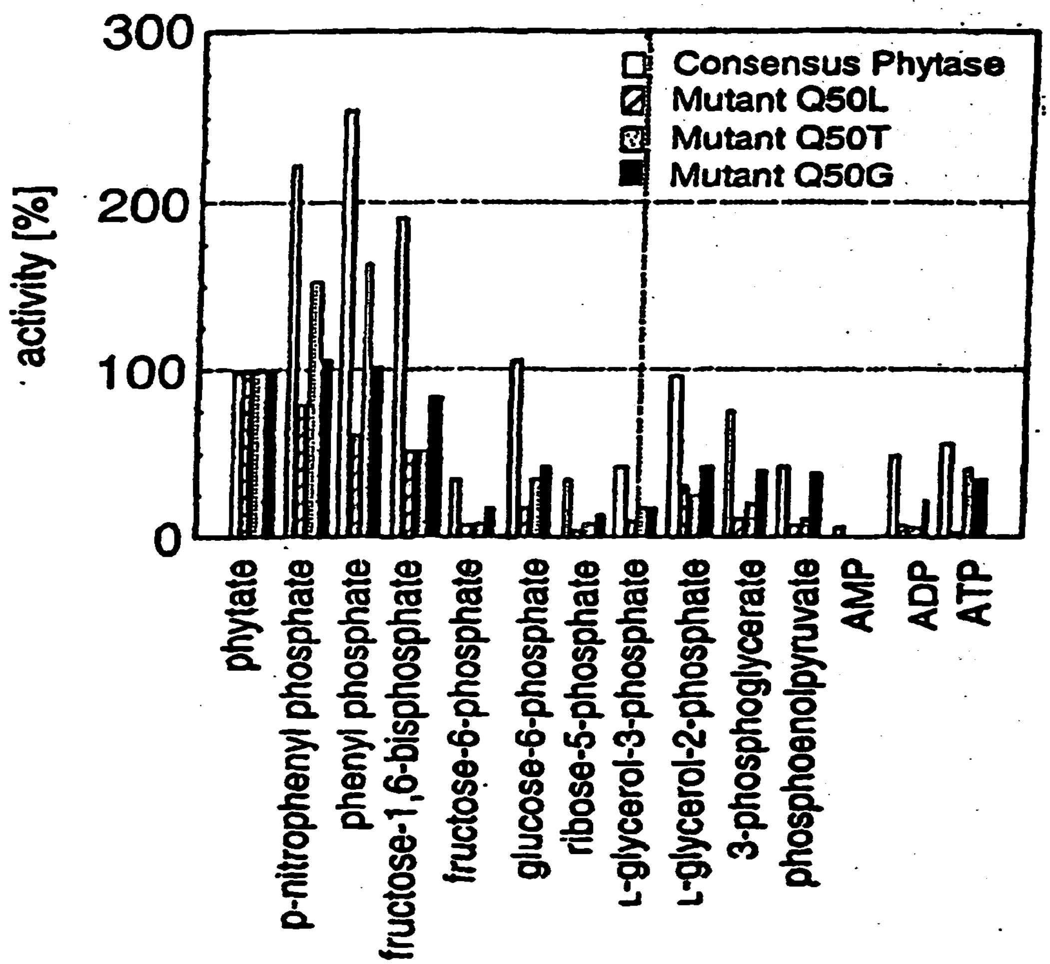
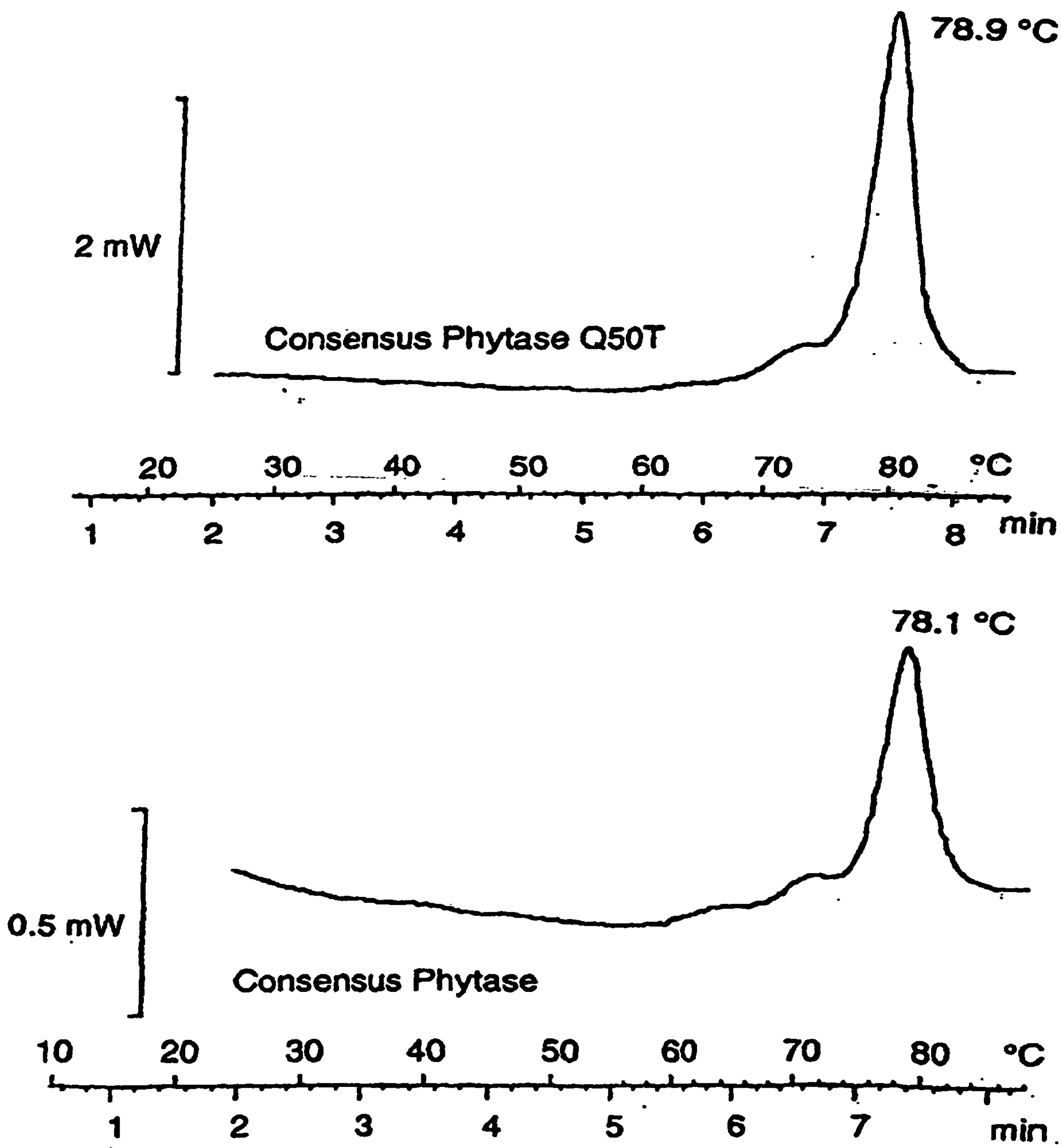

300
200
100
0
activity [%]
Consensus Phytase
Mutant Q50L
Mutant Q50T
Mutant Q50G
phytate
p-nitrophenyl phosphate
phenyl phosphate
fructose-1,6-bisphosphate
fructose-6-phosphate
glucose-6-phosphate
ribose-5-phosphate
L-glycerol-3-phosphate
L-glycerol-2-phosphate
3-phosphoglycerate
phosphoenolpyruvate
AMP
ADP
ATP

Figure 7


78.9 °C
2 mW
Consensus Phytase Q50T
20
30
40
50
60
70
80
°C
1
2
3
4
5
6
7
8
min
78.1 °C
0.5 mW
Consensus Phytase
10
20
30
40
50
60
70
80
°C
1
2
3
4
5
6
7
min